Can Epstein Barr Virus Infect Human Brain Cells In Culture?

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Abstract

Multiple Sclerosis (MS) is a chronic inflammatory disease of the Central Nervous System (CNS) with heterogeneous disease course, pathology and global distribution, though its aetiology remains unknown. There is evidence that genetic risk factors are involved, but the mode of inheritance is highly polygenetic and is probably responsible for producing a background of susceptibility in an individual. It is therefore likely that external factors influence the risk of MS development through gene-environment interaction and it is possible that infectious agents such as viruses could contribute to MS pathogenesis in this manner. Epstein Barr Virus (EBV) is a human herpesvirus that has been consistently linked with EBV throughout the last twenty years. EBV preferentially infects B cells and following infection persists in the host throughout the entire lifespan

The aim of this study was to successfully culture and infect epithelial cell lines and primary culture models of glial white matter and neuronal grey matter and examine the tropism of EBV through the use of PCR and RT-PCR techniques, and immunocytochemistry.

Our results show that EBNA1 protein expression is present in B cells as well as in epithelial cell lines RPE and HTB-9 and primary human mixed brain and neuronal cultures when they are exposed to cell-free EBV. There is also evidence that supports the production of different EBNA1 splice variants in the EBV infected human mixed brain cultures as compared to the B cell B95-8 EBV system. However, these are preliminary results that require further investigation.

In conclusion, these results suggest that EBV may be able to infect primary human brain cells in culture. This may support the hypothesis that EBV plays a role in the pathogenesis of MS and could contribute to the development of a human neurovirological model of MS in the future.

1. Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory disease of the Central Nervous System (CNS). The pathological hallmark is the presence of demyelinated plaques which often show predilection for the optic tracts, periventricular regions, brain stem and spinal cord (Hafler 2004). The disease begins typically between the ages of 20 and 40, and shows greater prevalence in women. Clinical symptoms include chronic fatigue and motor, sensory, visual and cognitive dysfunction and inevitable decline. Symptoms and clinical courses show great diversity between patients and are largely unpredicatable (Haahr & Hollsberg 2006).

Despite the fact that more than a century has passed since the first description of the clinical and pathological features of Multiple Sclerosis, the depth of its complexity means that there is no consensus on a single dominant aetiology. Research has focused on the pathogenesis of the disease and its progression in the hope of revealing the sequence of events that define the evolution of the characteristic inflammation. Although there is a frustrating lack of knowledge of the specific mechanisms involved, the presence of features distinct to the disease have facilitated the formation of valuable hypotheses about the aetiology of MS.

Genetics

The human leukocyte antigen (HLA) region is a gene locus that has been associated consistently with the disease. The presence of the HLA-DR1501 or HLA-DQ0601 alleles which encode restriction elements of T cells appear to substantially increase the risk of MS. At this time no other genes have been explicitly identified as being involved in MS (Hemmer et al. 2006), although it has been suggested that up to

eighty genes may be involved (Christensen 2006). This implies that the mode of inheritance is highly polygenetic.

Approximately 15-20% of patients that suffer have a family history of the condition (Hafler 2004), but the documented 'absolute' risk of the disease in a first degree relative is less than 5%. This still remains 20-40 times greater than the risk to the general population. There is a concordance rate of 31% between monozygotic twins, much greater than that of the identified 5% between dizygotic. (Noseworthy, Lucchinetti, Rodriguez, & Weinshenker 2000).

It appears from the evidence that genetic factors alone are probably insufficient to cause MS. It is more likely that they act as a 'background' of susceptibility and serve as a base upon which other factors could exert their influence through gene-environment interaction (Christensen 2006).

Epidemiology

The prevalence of MS differs notably around the world, with the highest (>30 per 100,000 individuals) in northern Europe, Southern Australia and the middle regions of North America. There is a current trend towards an increase in prevalence and incidence, with areas of note including southern Europe. In contrast, stable or declining rates are shown in areas of previously high prevalence and incidence, such as areas of Northern Europe (Cook 2002;Siegel 1997). Migration studies show that individuals that migrate from high-risk to low-risk areas after the age of 15 tend to take their MS risk with them, whereas those who do so before then acquire this lower risk (Lunemann et al. 2007).

Like the evidence for genetic predisposition, the reasons for these drastic differences in the epidemiology of MS are not well understood. It is probable that this unique

global distribution is due to a combination of several factors, from genetic origin of the population to environmental and social aspects of the area (Cook 2002). This has led to the belief that MS is likely to be the outcome of interplay between genetic predisposition and transmissible influences. It is these influences that have been receiving attention as the possible key to our understanding.

An association between viral infection and MS?

It has been suggested that infectious agents could act as transmissible influences, and therefore MS could be the result of a viral infection in genetically susceptible individuals. Although several viruses have been implicated in MS pathogenesis, such as coronaviruses, measles viruses and Chlamydia (Cook 2004), there is increasing evidence that the Epstein Barr Virus could be a viable candidate

1.2 Epstein Barr Virus (EBV)

EBV is a human herpesvirus. It is a large DNA virus associated with subclinical infection in young children and Infectious Mononucleosis (IM) in adolescents and adults (Cook 2002). Primary infection, either symptomatic or silent, usually occurs through salivary exchange and viral replication has been shown to occur in the epithelial cells of the nasopharynx and salivary glands. Following infection the virus persists in the healthy host throughout the entire lifespan (Cohen 2000).

EBV preferentially infects B lymphocytes through binding to the CD21 (CR2) receptor with MHC class II as its co-receptor (Dreyfus 2005;Jabs et al. 1999). EBV infected cells *in vivo* can express 4 different programs of gene usage dependent upon on the location and differentiation state of the infected B cell. The lytic stage is used to produce more infectious virus. In the 'growth' program all eight latent proteins are expressed and stimulate proliferation of the infected host B cell. The three latent proteins expressed in the 'default' programme are concerned with helping the

infected B cells survive. Lastly is the 'latency' programme, in which the virus persists in memory B cells without EBV protein expression (Niedobitek et al. 2000).

Amongst a total of more than 80 lytic and 8 latent EBV gene products, the latency-associated EBV nuclear antigen-1 (EBNA-1) is the only protein expressed consistently in infected proliferating memory B cells in healthy virus carriers. EBNA-1 has a role in initiating viral replication. It binds to the EBV circular DNA with its C-terminal domain and cross links the episome to mitotic chromosomes as a protein anchor, therefore accomplishing the transmission of the episome into progeny cells. EBNA1 is therefore crucial for the persistence of the virus (Cohen 2000) and thus a valuable indicator of infection.

EBV has been linked to several neurological conditions, those of note including diffuse or focal encephalitis, Guillain-Barre syndrome and chronic meningoencephalitis (Bray et al. 1992), and there is accumulating evidence that it could also be involved in MS.

1.3 EBV and MS

It has been many years since the possibility of a role for EBV infection in MS pathogenesis has been suggested (Warner & Carp 1981) though in the past, any findings that have linked EBV to MS have been attributed to altered host immunity (Larsen, Bloomer, & Bray 1985). EBV as a factor in the development and progression of the disease is still a contentious theory, but there are several observations of EBV infection that may offer explanation for the characteristic features of the disease.

Seropositivity

90% of the general population are seropositive for anti-EBV immunoglobulin G (IgG). In MS patients this statistic becomes nearly 100% (Christensen 2006). Although it appears paradoxical that a virus found in populations so ubiquitously could be to blame, this difference in seroprevalence between individuals with MS and those without is still significant. Seronegativity is associated with an extremely low risk of the disease (Ascherio & Munch 2000;Giovannoni & Ebers 2007).

Intrathecal Positivity

EBV DNA has been shown to be directly present in the cerebrospinal fluid (CSF) of MS patients (Menet et al. 1999), and oligoclonal bands (OCBs) are frequently found in individuals with the disease (Cepok et al. 2005a;Correale & de los Milagros Bassani Molinas 2002). Although the OCBs in MS are polyspecific, the use of a human cDNA protein expression array revealed that the two most frequent MS specific and high affinity epitopes are both derived from EBV. These are EBNA1 and the structural EBV protein BRRF2. Rand et al (2000) present a subset of patients with a strong oligoclonal CSF Antibody (Ab) reactivity to EBV nuclear antigen type 1 (EBNA-1). However, this is not true in the majority of patients as, although EBV seropositivity is close to 100% in MS patients, only around 30% have an intrathecal Ab response to EBNA-1 (Cepok et al. 2005b).

Prior Infection and Infectious Mononucleosis

Patient cohort longitudinal studies suggest that prior infection with EBV, as assessed by the presence of elevated anti-EBNA1 Ab more than 10 years before the occurrence of symptoms, is associated with an increased risk of developing MS (Lindberg et al. 1991;Nielsen et al. 2007).

The timing of this prior infection appears important as several studies indicate that many MS patients have had measles, EBV or other childhood infectious diseases at

a later age than control individuals. A primary EBV infection in early childhood is often not associated with the symptoms of IM, but when delayed until adolescence this event results in IM in many more cases (Warner & Carp 1981). An increased risk of MS development correlates with this later EBV infection, and therefore individuals with a history of IM have a more than twofold increase in their risk in comparison to control individuals who acquired the virus without symptoms (Christensen 2006;Nielsen, Rostgaard, Nielsen, Koch-Henriksen, Haahr, Sorensen, & Hjalgrim 2007). As a result there is a marked epidemiological relationship between the two (Lindberg, Andersen, Vahlne, Dalton, & Runmarker 1991).

Epidemiology

Ascherio's 2000 review analysed relevant published articles about the link between the presence of antibodies against EBV and the risk of MS. Upon evaluation, and exclusion of spurious results, they concluded that a causal relation between EBV and MS would fit a wealth of epidemiological data, including the geographical distribution of the disease. For example, the fact that regions of higher socio-economic status are associated with a higher prevalence of MS could be explained by the 'hygiene hypothesis'. This states that the immune system needs to be challenged early in life in order to develop successfully. In these more sterile environments there is decreased exposure to infectious agents such as EBV. This is supported by the trend that EBV prevalence in prepubescent children is lower in regions where MS is common. In the less sterile areas of lower socio-economic status infection with EBV will occur at a younger age due to the lack of protection, and is therefore likely to be both subclinical and to offer immunization (Giovannoni & Ebers 2007;Haahr & Hollsberg 2006).

Disease activity and viral reactivation:

Periodic reactivation is a characteristic property of herpesviruses, and a possible involvement in the pathogenesis would account for the relapsing-remitting course that is usually found in early stages of the disease (Wandinger 2000). However, one study showed no conclusive relationship between the time course of the disease and the biology of EBV. This suggests that replication of the virus may be invloved in some manner in the pathogenesis, though not the progression, of MS (Buljevac et al. 2005). This is supported by the fact that in the first few years after onset there are a significantly higher number of EBV reactivations, coinciding with the clinical point at which the disease is most active (Christensen 2006;Levin et al. 2005)

Molecular Mimicry

Molecular mimicry is an immune phenomenon that has been suggested as a mechanism of MS pathogenesis. Epitope similarity between microbial and self-antigens leads to the activation of autoreactive T-cells, providing a link between autoimmunity and viral infection. A potential for mediating a break in tolerance for self-antigens appears to be a key factor in MS development and, given the life long persistence of EBV and the possibility of its periodic reactivation, the virus has all features required for a sustained cross-reactive autoimmune response (Pohl et al. 2006).

Evidence that supports EBV involvement on this mechanistic level is the recognition that EBNA1 virus derived peptide sequences are stimulatory for a myelin basic protein (MBP) specific CD4+ T cell clone (Bray, Luka, Bray, Culp, & Schlight 1992;Larsen, Bloomer, & Bray 1985). However, a definite positive link between EBV reactivation and disease activity in MS patients over time would provide further support for this theory.

1.4 The EBV Infection of CNS specific cells

Demyelination and axonal injuries are the result of a complex sequence of events that includes processes intrinsic to the CNS. This means that MS is not a just a disease of the immune system, but of CNS specific components.(Wandinger et al. 2000). However convincing the current observations may be of a link between EBV and MS, there is still a lack of direct evidence for the infection of brain cells with the virus.

The presence of EBV in the CSF suggests that the virus can get in contact with neural cells (Menet, Speth, Larcher, Prodinger, Schwendinger, Chan, Jager, Schwarzmann, Recheis, Fontaine, & Dierich 1999) and although it is possible that this phenomenon is largely due to the presence of latently infected B lymphocytes in the brain it is still feasible that successful infection of brain cells could occur.

Strong evidence in support of EBV infection of CNS tissue is the detection of EBV DNA in brain biopsy samples from individuals suffering from neurological conditions in which EBV is suspected to play a role (Menet, Speth, Larcher, Prodinger, Schwendinger, Chan, Jager, Schwarzmann, Recheis, Fontaine, & Dierich 1999). However, there is a lack of evidence of EBV in post mortem samples. In a study where mRNA presence was assessed no difference was seen between control and MS brains (Opsahl & Kennedy 2007). Therefore it remains difficult to prove or disprove that EBV is capable of the infection of CNS specific cells as attempts to identify the EBV genome in MS brain tissue have either been negative or non-specifically positive (Cook 2002).

Menet et al (1999) have shown successful *in vitro* infection of astrocytes, the immunologically active cells in the CNS. Although they used a cell line transfected with the CR2 receptor they obtained the same transcription pattern as seen in wildtype primary astrocyte cultures. At this time there has been no expansion upon

this work to include other cells found in the brain. Although Ascherio et al (2000) argue that the available epidemiological data is sufficient to justify a causal relationship between EBV and MS, and thus mechanistic understanding is unnecessary, a model that demonstrated the tropism of the virus in the cells of the CNS would prove invaluable in the case to implicate EBV and to study the infection.

The aim of the study

The aim of this study was to successfully culture and infect epithelial cell lines and primary neuronal cultures and examine the tropism of EBV in each and how it differs.

B95-8 is a marmoset B cell line that secretes EBV. This is a well characterized model that will serve as the positive control in this study. B95-8 was originally immortalized through infection with EBV from the 883L cell line obtained by culture of lymphocytes from an elderly individual with transfusion produced IM. The B95-8 strain of EBV is not a naturally occurring isolate, but rather a laboratory-adapted variant (Skare et al. 1982). In comparison with human cord leukocytes, this marmoset leukocyte line was found to yield a considerably greater quantity of active virus and therefore serves extremely useful as a productive source of EBV (Miller & Lipman 1973).

Tetradecanoyl phorbol acetate (TPA) can be used to induce the lytic cycle in the B-95-8 cells. Zp is the promoter of the EBV immediate early gene BZLF1, and Zp has several response elements sensitive to TPA. The product of BZLF1 is ZEBRA, which drives the lytic cycle in B958 via a protein kinase C-dependent mechanism (Davies et al. 1991;Gradoville et al. 2002). This method can be used to produce two populations of B95-8 cells, one of which will be more virally productive due to this TPA stimulation. During the viral productive cycle the EBV genome is amplified approximately 100 times (Kudoh et al. 2003).

Retinal Pigment Epithelium (RPE) is an adherent cell line derived from the normal eyes of a 19 year old male who died from head trauma in a motor vehicle accident. It is a rapidly growing, spontaneously evolved human cell line. They possess defined cell borders, and overall 'cobblestone' appearance and noticeable pigmentation (Dunn et al. 1996). Human Bladder Carcinoma (HTB9) is an adherent cell line originally sourced from a grade II tumour of a 68 year old Caucasian male. Both cell lines demonstrate prototypical features of human epithelia in culture.

The goal was to harvest EBV from stimulated B958 cultures and infect these epithelial cell lines. This infection would be followed over a period of 14 days. At days 1, 3, 6/7 and 14 post infection cell lysates were collected from the infected and control samples. The success of the infection was assessed and characterized through the extraction of DNA and RNA from these lysates. Identification of EBV specific DNA and RNA transcripts was achieved by polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR). Both latent and lytic genes were investigated, including the persistently expressed nuclear envelope protein **EBNA1.** **LMP1** is a latent membrane protein, involved in the suppression of apoptosis (Thorley-Lawson & Babcock 1999;Wang, Rowe, & Lundgren 1996). Immediate early lytic gene **BZLF1** stimulates a cascade that results in the initiation of the viral productive cycle (Kudoh, Fujita, Kiyono, Kuzushima, Sugaya, Izuta, Nishiyama, & Tsurumi 2003). **gp350** is a glycoprotein found on the viral envelope of lytically infected cells only (Savard et al. 2000). This provides an indication of the expression profile of the virus in these cell types. In addition an accompanying infection time series was established for each culture type to be used specifically for immunocytochemistry.

Cell free virus was collected from the supernatant of the B95-8, RPE and HTB-9 cell cultures, providing a source of EBV from three different cell lines which could be

used to infect the primary brain cell cultures. Primary mixed brain cultures consist of approximately 70-80 % astrocytes, 20% neurones, ~5% oligodendrocytes and 1% microglia, and can be used as a model of glial white matter. The primary neuronal cultures (70-80% neurons, 20% astrocytes, 5% oligodendrocytes) can be used as a model of grey matter (Trillo-Pazos et al. 2000).

Each primary culture was infected with EBV derived from either B958 or infected RPE and HTB-9. As with the epithelial cell lines, the infection was followed in each primary culture system over 14 days. At days 1, 3, 6/7 and 14 post infection cell lysates were collected from the infected and control flasks for analysis. Following DNA and RNA isolation from the lysates of these time series, PCR and RT-PCR techniques were used to judge the EBV DNA content of the cultures, and to distinguish the expression pattern of EBV transcripts in them. These results can then be compared to those for the B958 positive control and then for the epithelial cells. Cultures established for immunostaining will provide further evidence of the infection, as well as allowing visualization of the effects of infection in these CNS matter models.

2. Materials and Methods

2.1 Cell Culture

Materials and Techniques

DMEM F12 advanced medium (GIBCO) was used for the culture of epithelial cell lines and mixed brain cultures. RPMI 1640 medium (GIBCO) was used for the culture of B lymphocytes (B958). These include non-essential amino acids and sodium pyruvate. 40 ug/ml Gentamycin sulphate, 10%-2.5% fetal bovine serum (FBS) and glutamine were added. Different FBS concentration were used depending on cell type. Once cells were established, sera was lowered so as to mitigate proliferation and increase differentiation within the culture, a 1% solution was preferable to the initial 5% used when setting up the cultures. Mixed brain cultures were cultured in DMEM F12 advanced medium with 5% FBS. Chemically defined Neuronal Base Medium (PAA- U15-023) was used for the neuronal cultures. Sterile Hank's Balanced Salt Solution (GIBCO) was used for all washing of cell cultures.

Passaging

Once >90% confluent cells were split at a ratio according to their growth rate e.g 1:4, 1:8 etc. 0.25% trypsin + EDTA protease solution was used to detach the adherent epithelial cells resulting in a cell suspension. Cells in suspension were centrifuged at 1500 rpm for 5 minutes to create a cell pellet, and this split into the appropriate number of flasks.

Cell Lysate collection

The medium was removed from flasks, cells washed 2-3 times in HBSS to remove bound serum. 2-3mls of HBSS was then added and the cells scraped from the surface into suspension. The suspension was then pipetted into 1.5ml eppendorfs. To pellet lysates cells were centrifugued at 5000-10000rpm for 30sec – 1 min. The supernatant was then discarded and the lysate transferred to -80C freezer.

Virus Collection Universal tubes containing the cell supernatant are centrifuged at 15,000 rpm for 2hrs at 4C. The viral pellet was resuspended in 3-4 mls HBSS, transferred into 1.5ml cryotubes and stored at -80C.

Cell lines

Epthelial and lymphocyte cells were cultured from frozen stock, where they were suspended in cryoprotectant freezing medium (90%FCS, 10% DMSO) at a concentration of 5 $x10^6$/ml.

B95-8

B95-8 cells were cultured in suspension and were seeded into 2x T75 flasks into 25mls RPMI. The flasks were placed into an incubator at 37C and 5% CO_2. In order to create a second population of TPA stimulated B95-8, one of the flasks was exposed to 100 ng/ml of TPA. The cell-free EBV was collected from the supernatant of these stimulated B95-8 cells after 48 hrs as described above.

RPE and HTB-9

The epithelial cells were seeded into T75 flasks at a concentration of approx $2.5x10^6$ in 12mls of DMEM/F12 medium. Cells were passaged once >90% confluent, and the culture medium was changed every 3-4 days.

Infection of Epithelial Cells

Once established, epithelial cell cultures were infected with the EBV collected from the concentrated stock (1:100) of TPA stimulated B958 cells. Infection occurred overnight at a 1:100 dilution and was monitored for 14 days. At days 1, 3, 6/7 and 14 post infection cell lysates were taken. EBV was collected from the epithelial cell line supernatant at day 21 post- infection, as described above.

Primary Cultures

Mixed Brain Cultures (70-80 % astrocytes, 20% neurones, ~5% oligodendrocytes and 1% microglia)

Foetal brain tissue was kindly provided by University College Hospital clinical terminations with ethical committee approval for the project. The tissue was placed in culture no later than 2-4 hours after clinical collection. The tissue was collected onto holding medium (HM), which contains: modified HBSS without calcium chloride and magnesium sulphate; 2mM glutamine, 10mM HEPES buffer, 20 µg/ml gentamycin sulphate. The tissue meninges were removed and the tissue washed 3-4 times in HM, then mechanically disaggregated. This involved separation with a scalpel and passage through a Pasteur pipette. This tissue suspension was left to stand for 5-10 minutes to allow the largest of the tissue clusters to settle to the bottom of the universal. This was then passed through a 200µM Nylon mesh.

To prepare the mixed cultures this suspension was diluted into $25cm^3$ flasks in DMEM/F12 at 5% FCS at a concentration of 2×10^5 cells/ml and some cells were cultured on glass coverslips coated with poly-D-lysine in 12-well plates for later immunostaining. Cultures were left to mature for two weeks. NMDA receptor expression was used as a indication of culture maturation (Trillo-Pazos et al, 2000). Media was changed every 3-4 days.

Neuronal Cultures (70-80% neurons, 20% astrocytes, 5% oligodendrocytes)

Mechanical disaggregation, as above, was used to prepare neuronal cultures and were seeded into chemically defined media (Neruobasal and B27) so as to minimize growth of glial cells in the culture. For staining purposes neuronal cells were seeded onto glass coverslips coated with poly-D-lysine (0.1. mg/ml) and Laminin (5 ug/ml).

Cultures were left to mature for four weeks as assessed by NMDA receptor expression (Trillo-Pazos et al, 2000). Media was changed every 3-4 days.

Infection of Primary Cultures- Mixed Cultures and Neuronal Cultures

Primary cell cultures were infected overnight with EBV at 1:100 derived from concentrated stock of EBV grown from B958, RPE or HTB9 cell sources as described. Following this the virus was washed out twice with HBSS and the medium replaced. At days 1, 3, 7 and 14 post infection cell lysates were taken from the mixed brain and neuronal cultures, as described.

2.2 Molecular Biology

DNA extraction- using DNeasy mini kit (QIAGEN)

DNA extraction was performed according to QIAGEN DNeasy kit instructions.
A proteinase is added to the lysate to break down the cell membranes. A DNeasy spin column is then used to extract the DNA from the solution. This utilizes a silica-gel membrane to filter the solution. The membrane binds the RNA and contaminants are washed away by the application of several buffer solutions and centrifuge steps. A final elution step with sterile RNase free water results in a DNA solution. This solution is stored at -80C, since DNA stored in water is subject to acid hydrolysis.

DNA/ RNA quantification- this was performed using the 'Nanodrop' ND-1000 system, a spectrophotometer that can measure either DNA or RNA based on loading 1 µl of the eluted sample solution.

PCR- using HotStar Taq DNA polymerase (QIAGEN)

PCR reagents: The volume of reagents used is dependent upon the concentration of template DNA to be used (as assessed with the Nanodrop), the volume of which will differ from sample to sample. The aim was to use between 500ng and 1µg per reaction. A volume of master mix of reagents is prepared 10% greater than the volume required for the number of reactions. This provides a sample of the mix which can be included as a negative control to eliminate a source of contamination.

Table 1: PCR reagents

Component	Volume/reaction	Concentration
10 x PCR buffer	2.5 µl	1 x
dNTPs (10mM each)	0.5 µl	200 µM each
MgCl2	0.5 µl	2.0 µM
Primer (forward)	1.0 µl	-
Primer (reverse)	1.0 µl	-
HotStar Taq	0.25 µl	2.5 units/reaction
DNase free water	Variable	-
Template DNA	Variable	-
Total volume per reaction	25µl	

Primer and PCR programe design:

See table 2 for Primers and PCR programmes.

A ubiquitously expressed 'housekeeping' gene is selected for amplification as a control. β-globin is the housekeeping gene.

HotStarTaq DNA polymerase is provided in an inactive state, and is activated by a 95C 15 min incubation step as a prelude to the PCR programme. The extension rate of the polymerase is 2-4kb per minute at 72C. The size of the amplicon therefore dictates the length of this step. The primers themselves are responsible for the temperature of the annealing step. The number of cycles are dependent upon the no. of copies of the starting template DNA. The number of PCR cycles generally used is between 25-35. An excessive number of cycles will likely lead to smearing and amplification of a non-specific product.

In all PCR programmes used there is an initial step of 95C for 15 min to activate the polymerase. Following the end of the cycle loops there is a further extension of amplicon at 72C for 10 min, and a final stage of 8C for 15 min.

Protocol

Reaction mixture is prepared according to directions (HotStarTaq PCR Handbook 2005 ed.), with the exception of the HotStarTaq polymerase. The desired volume of template DNA is added to each 0.2 ml PCR tube. The polymerase can then be added to the

Table 3: Primer Design and PCR conditions

PRIMER	SEQUENCE (5' TO 3')	%GC	SIZE	Annealing Temp	Duration	No. Cycles
B-GLOBIN 3	CAA CTT CAT CCA CGT TCA CC	50	267	50	1 min	30-40
B-GLOBIN 4	GAA GAG CCA AGG ACA GGT AC	55				
EBNA1 R	GAC CAA AGG TGG ATA CCA CCT TCC	54	1795	58	1min 30sec	40
EBNA1 F	GGA GAC ACA TCT GGA CCA GAA G	54				
EBNA1Q R	AGC GTG CGC TAC CGG AT	64	220	55	1 min	40
EBNA1Q F	TGG CCC CTC GTC AGA CAT GAT T	54				
GP350 R	GTA ACG TAC ACC CAT TTT ACC CT	43	2582	56	2 min	40
GP350 F	TAT GGG ATG TAG ACA AGT TAC GCC	45				
BZLF1 R	ATA GCA AAG GTG GCC GGC AAG GTG C	60	478	63	1 min	40
BZLF1 F	TGT GGA ACA CCA ATG TCT GCT AGC	50				
LMP1 R	AAG AGG AGG AGA AGG AGA GCA AGG	50	1167	60	1min 30 sec	40
LMP1 F	AAA AGC AGC GTA GGA AGG TGT GGA	50				
GAPDH R	TGG TGA AGA CGC CAG TGG AC	50	268	56	45 sec	25
GAPDH F	ATG GGG AAG GTG AAG GTG GG	50				
BFRF1 R	GGC CCA GCA TAA TGA TGG AGT	48	366	50	1 min	30
BFRF1 F	AGA GGC TCC TAG ACG AGC TC	60				
BZLF1 R (alt.)	ATT GTC TCC AGG TTG AGG TG	50	347	50	1 min	30
BZLF1 F (alt.)	ATG TAG GAC CCA AAC TCG AC	50				
BXLF2 R	GGA ATT CAG GCC GCT AAT GA	50	353	50	1 min	30
BXLF2 F	GGA TGC AGT TGC TCT GTG TT	50				

BFRF1, BZLF1 (alt.) and BXLF2 were designed and kindly donated by Dr E Tsao (UCL department of Infection and Immunity)
All other primers were designed by Dr G Trillo-Pazos and ordered from Invitrogen

reaction mixture, and this added to each PCR tube. Each tube can then be placed into the thermal cycler. Following cycle completion the amplified DNA product is kept at -20C.

RNA extraction- using RNeasy mini kit (QIAGEN).

RNA extraction was performed according to QIAGEN RNeasy kit instructions (2006 ed.) Samples are lysed and homogenized in the presence of a denaturing buffer which inactivates RNases to ensure extraction of intact RNA. A spin column is used for the extraction process. The silica-gel membrane of the spin column binds the RNA and contaminants are washed away through the application of buffer solutions and successive microcentrifuge steps. Although the membrane is designed to remove most of the DNA, an additional DNase treatment stage was used. For the elution step RNase-free water is used, and the resulting RNA solution transferred to -80C freezer.

One step RT-PCR using QIAGEN OneStep RT-PCR kit

One step RT-PCR Reagents

As with PCR, the volume of reagents used is dependent upon the concentration of template RNA to be used (as assessed with the Nanodrop), the volume of which will differ from sample to sample. A volume of master mix of reagents is prepared 10% greater than the volume required for the number of reactions.

Table 3: One Step RT-PCR reagents

Component	Volume/ reaction	Final Concentration
5 x OneStep RT-PCR Buffer	10.0 µl	1x
dNTP mix (containing 10mM each)	2.0 µl	400µM each dNTP
Primer (forward)	1.0 µl	0.6µM
Primer (reverse)	1.0 µl	0.6µM
OneStep RT-PCR enzyme	2.0 µl	-
RNase Inhibitor	0.25 µl	5 units per reaction
RNase-free water	Variable	-
RNA template	Variable	-
Total volume per reaction	50 µl	-

One Step RT-PCR Primer and Programmes

The same primers can be used as are in the PCR reactions, but the 'housekeeping' role of β-globin has been replaced by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also ubiquitously expressed.

The same RT-PCR programme rationale is used as in the case of PCRs. Therefore the same programmes on the cycler are used but with the addition of a 35 minute step of 50C at the beginning for the RT step.

One Step RT-PCR Protocol

Reaction mixture is prepared according to directions (OneStep RT-PCR Handbook 2005 ed.), with the exception of the RT-PCR enzyme. The desired volume of template RNA is added to each 0.2 ml PCR tube. The enzyme can then be added to the reaction mixture, and this added to each PCR tube. Each tube can then be placed into the thermal cycler. Following cycle completion the amplified cDNA product is kept at -20C.

Two step RT-PCR- using the Roche Transcriptor First Strand cDNA Kit.

This works upon the same basis as the One-step PCR, but instead of reverse transcription and amplification occurring in one programme, this technique separates them into two separate steps. If the yield of RNA from a sample is small this process allows us to ultimately use less of the sample.

RT stage reagents: As previously, the volume of reagents used is dependent upon the concentration of template DNA to be used (as assessed with the Nanodrop), the volume of which will differ from sample to sample. A volume of master mix of reagents is prepared. This volume is 10% greater than the volume required for the number of reactions.

Table 4: Two step RT stage reagents

Component	Volume/reaction	Final Concentration
Step 1: Denaturing Step (vol per reaction = 13µl)		
Template RNA	1 µg but can vary dependent on overall yield	
Primer (anchored oligo[dT])	1 µl	2.5µM
RNase free water	Varies according to RNA concentration	
Step 2: Reverse Transcription		
Reverse Transcriptase Buffer x 5	4 µl	1 x
Protector RNase Inhibitor	0.5 µl	20U
dNTPs (10mM each)	2 µl	1mM each
Transcriptor reverse transcriptase	0.5 µl	10 U
Total Volume per reaction =	**20µl**	

RT stage- Primers: The use of anchored oligo[dT]18 primers was suitable as it binds from the very beginning of the poly[A] tail, generating full length cDNA.

RT stage protocol

There are two stages to this- the denaturing step and then the reverse transcription. First the template RNA-primer mixture is prepared, as detailed above, per reaction for the denaturing step. This is then place in a heated PCR cycler at 65C for 10 min.

Follows this cycle the tubes are placed on ice. The remaining reagents are added and the template RNA-reaction mixture can be incubated at 55C for 30 min in a heated PCR cycler. At the end of this 30 min is a 5 min 85C step to inactivate the enzymes. This resulting cDNA from this RT stage can then be kept at -20C and used for the PCR stage without purification (see **3.1** for details of PCR protocol using HotStarTaq polymerase).

Gel Electrophoresis-

5 µL of the sample was loaded into each well of a 1.5% agarose gel (5ug/ml Ethidium Bromide) using X6 loading buffer. The molecular Marker used to correlate the size separation was the Hyperladder I (BioLine), which covers fragment sizes of 200bp to 10Kb, or Hyperladder II (BioLine), which covers a range of 50bp to 2Kb.

2.3 Immunocytochemistry-

Both single and double labelling staining was attempted. Single labelling uses a single specific antibody and an accompanying nuclear counterstain, whereas double labelling requires two antibodies. This allows the successful visualization of co-localized target antigens. In order to strengthen the signal differing concentrations of antibody were tried. However, a compromise was made through the use of a species specific biotin (VECTOR LabInc). This is used to amplify the signal from the bound Ab.

Table 4: Antibodies (all used at 1:100 dilution in PBS)

Culture	Antibody (species)	Target
B958	CD20 (mouse)	B Cell surface receptor
	CD68 (mouse)	Macrophage surface glycoprotein
	EBNA1 (goat)	Nuclear Ag- EBV
	CD21 (mouse)	EBV receptor
RPE and HTB9	Pancytokeratin (mouse)	Cytoskeleton- epithelial cells
	EBNA1 (goat)	EBV
	CD21 (mouse)	EBV receptor
Mixed Brain Culture	GFAP (mouse)	Cytoskeletal protein- Astrocytes
	Tubulin III (mouse)	Cytoskeletal protein- Neurons
	CD68 (mouse)	Macrophages/ microglia
	CD20 (mouse)	B cells
	CNPase (mouse)	Oligodendrocytes
	CD21 (mouse)	EBV receptor
Neuronal Cultures	MAP2 (mouse)	Microtubules- neuronal dendrites
	MAG (mouse)	Myelin- oligodendrocytes
	GFAP (rabbit)	Cytoskeletal protein- astrocytes
CD21, CD68 and GFAP all from DakoCytomation, all other Abs from SIGMA		

2.1.1 Staining protocol

The B lymphocytes had to be fixed at a suitable concentration to allow a clear view for staining. This required them to be left on the slide to 'dry'.

Epithelial and primary cultures are grown on coverslips in wells specifically for the goal of staining. At the appropriate time point (d1, 3, 6/ 7, 14) these were taken and stained for the appropriate antibodies.

Cells were fixed using an acid/alcohol solution of 1%HCl/ 99%EtOH, 'blocked' using 1% Horse Donor Serum (HDS) and the primary antibody was applied at 1:100 dilution for 30 mins. Following this the antibody is washed off three times with PBS.

The species appropriate biotin is added at 1:100 dilution in PBS and left for 30 mins. The flourochrome, Alexa 488 (FITC), was then applied at 1:200 dilution in PBS for 30mins. If double immunostaining the cells the next primary Ab, from a different species, is added as before and labelled with the flourochrome Alexa 568 (TRITC). The slides were then mounted using VectaShield mountant (VECTOR LabInc) containing DAP1, a nuclear counterstain. Slides must then be stored in the fridge, in the dark.

All images were taken using a Zeiss fluorescence microscope and Axiovert imaging software.

3. Results

3.1 B lymphocyte cell line B95-8 infected with EBV.

B95-8 cells express CD20, CD38 and EBNA1, both in the latent and lytic states **(fig 3.1)**. Positive staining for CD20 identifies B cells, whereas CD38 indicates cell activation. As would be expected a stronger signal is present for EBNA1 **(F)** when cells are activated after treatment with TPA for 48 hours compared to unstimulated. There is a lesser increase apparent in the signal for immunostaining of the CD20 receptor **(B).** There is also a higher background non-specific stain **(H)** in the activated cells than the inactivated. These results demonstrate that B95-8 cells are of B cell lineage and express EBV in a latent and lytic stage upon activation.

The PCR results of stimulated B958 serial dilutions give a representation of the DNA concentration required for the primers to produce detectable amplified PCR products (fig 3.2D). Their results can also be used to construct a standard curve. If the gene is detected in other cell lines this curve can provide a means of speculative quantification. We could successfully detect latent (EBNA1, LMP1) as well as lytic (gp350) genes associated with EBV replication in this cell line (fig3.2). The EBNA1 primer taken from Lieberman (2005) (EBNA1Q) produces a faint band at the expected size of 220bp at a minimum concentration of 10^3 cells (fig3.2A), though there is several other bands of different sizes consistently present to a minimum concentration of 100 cells. These PCR results confirm that EBV is present in our positive control cell line of B958.

β-globin primers produce a result at 10^3 for the expected amplicon of 268bp (fig 3.2B). The primers designed for EBNA1 (fig3.2B), LMP1 (fig3.2D) and gp350 (fig3.2E) produce no fragments at the anticipated sizes, which are 1,795bp for EBNA1, 1,167 bp for LMP1 and 2,582 bp for gp350. However, there is evidence of

Figure 3.1: EBV infection of unstimulated abd stimulated B95-8

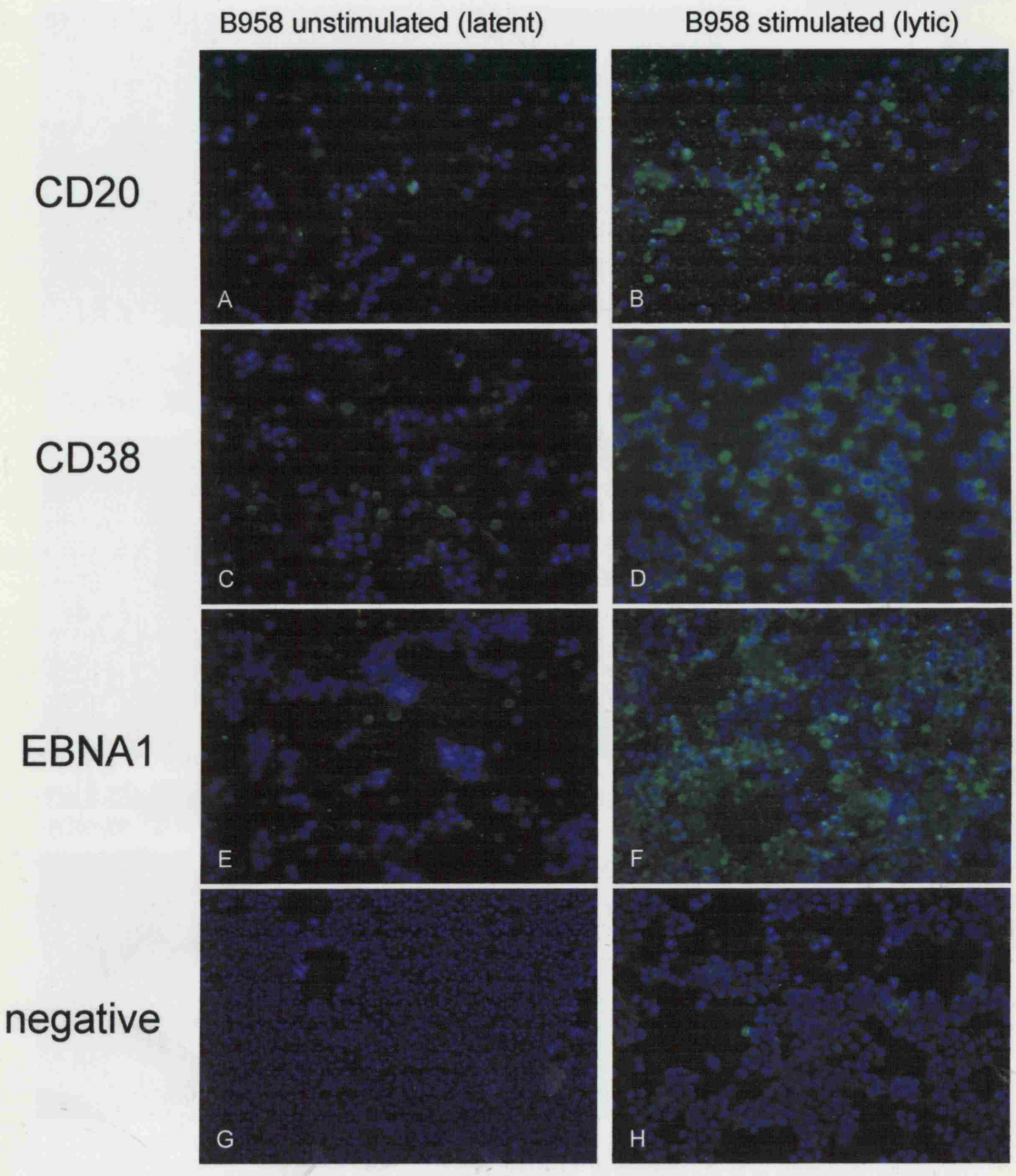
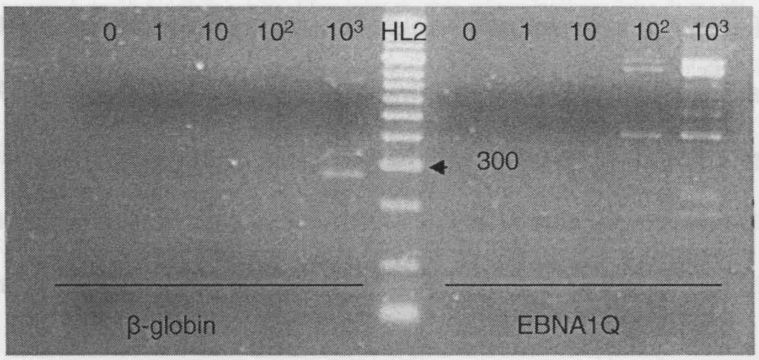


Figure 3.1: Detection of surface and nuclear antigens of TPA stimulated and unstimulated B958 cells. The most marked difference upon stimulation of the cells with TPA is the obvious increase in signal for EBNA1 **(E vs F).** There is also an increase in signal in the stimulated cells for the CD20 and CD38 Abs **(A vs B)** and **(C vs D).** The negative controls **(G)** and **(H)** are minus primary Ab.

Fig 3.2A: PCR gel of a serial dilution of stimulated B958



BioLine Hyperladder I (HL1): 200bp- 10Kb

BioLine Hyperladder II (HL2): 50bp – 2kB

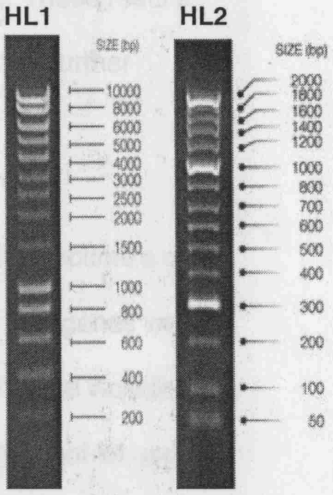


Fig 3.2B: PCR gel of a serial dilution of stimulated B958 using different EBNA-1 primers

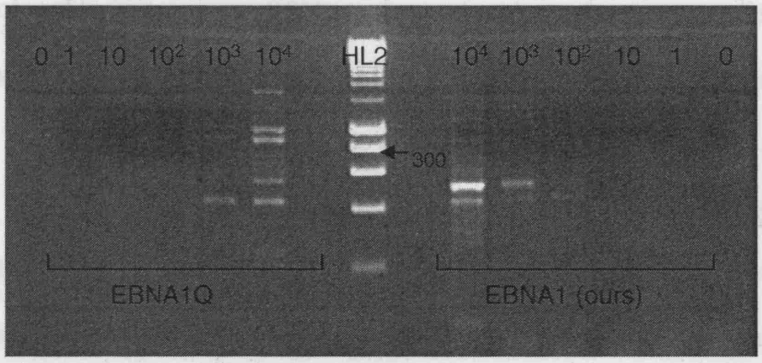


Fig 3.2 C, D and E: PCR results of a serial dilution of stimulated B958 with EBV specific primers

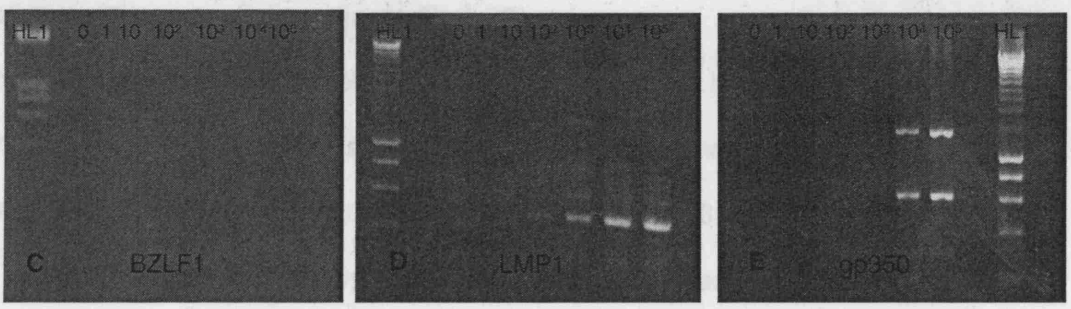


Figure 3.2: serial dilutions of stimulated B958- (A) The lowest limit of detection of the 267 bp β-globin amplicon using our primers and PCR programme was 1000 cells. The EBNA1 primer used is taken from Lieberman (2005) and is specific for a 220bp amplicon which can be seen at 1000 cells. No contamination was apparent in either of these PCR reaction mixtures, as seen by the absence of non-specific bands in the '0' lane which contains the reagent mix without the PCR product DNA. **(B)** The EBNA1 primer of our own design was as sensitive as that published in the literature. **(C)** No results were seen at all from the use of the BZLF1 primer, but no contamination is apparent. **(D)** The primer for LMP1 showed weak bands at the specified size of 1167bp. The '0' lane shows no reagent contamination. **(E)** The gp350 results show no band at 2582bp.

other amplified spliced products. For example, gp350 results demonstrate two bands (top at >1,500 bp and lower at >600-800 bp) that together are of the size of the expected amplicon (2,500 bp) (Fig.3.2F). These types of multiple splicing mechanisms of the EBV genome have being demonstrated in previous studies (Nonkwelo et al 1996). BZLF1 produced no result at all (fig3.2C). However, it is known that BZLF1 is expressed and degraded quickly upon EBV stimulation into a lytic mode of replication and has short half life. These results demonstrate different sized spliced variants than those expected and warrant further investigation.

Investigating the sources of error in the PCR protocol. In order to further optimize our PCR protocol to improve our EBV amplicons in this system, other primers and genes were tested within this B95-8 cell line. Possible sources of error examined at this time included the primers in use, the temperature of the annealing step of the protocol, the reagent kit used and the DNA. The 'alternative' reagent kit 'GoTaq' contained a Taq polymerase that did not require the activating 94C step. Although β-globin primers produced results at all temperatures and with both reagent kits, EBNA1 did not (fig 3.3A). The same experimental design was repeated with the LMP1 and BZLF1 primers of our own design, neither of which produced any of the expected amplicons (fig 3.3B).

Temperature gradient PCR was used to test the primers and the reagents at differing annealing temperatures. The same reaction occurs, with the same DNA, reagents, primers etc but at differing temperatures, in this case 50, 52, 55, 61, 65, 68 degrees celsius (fig 3.3 C and D). This is used to assess the optimum temperature for the annealing step when all other steps in the PCR programme remain the same. LMP1 was tested again using the temperature gradient and both reagent kits and again produced no results (fig 3.3 D). The only positive result from these was the result for an alternative BZLF1 primer (donated by Dr E Tsao) which was later shown to be

Fig 3.3: Examining the possible sources of error in PCR.

Fig 3.3 A: Different reagent set and annealing step temperatures- HotStarTaq vs Taq PCR results for B958. The step temperatures (degrees celsius) are indicated on the image

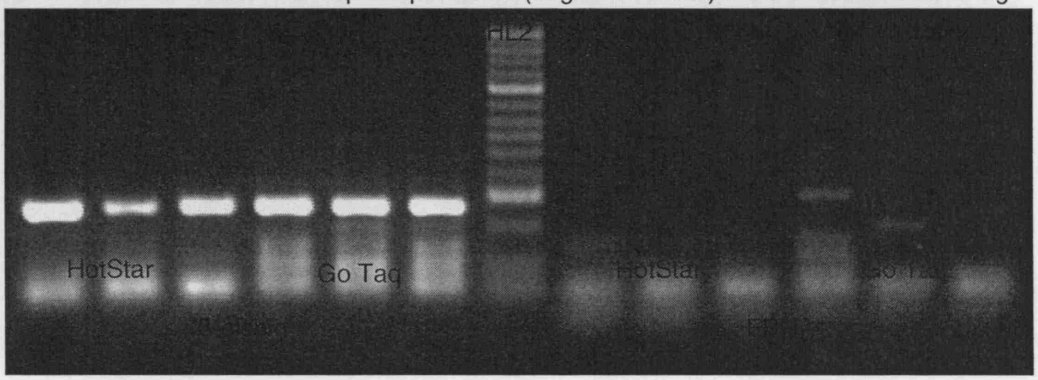


Fig 3.3 B: Different reagent set and annealing step temperatures- HotStarTaq vs Taq PCR results for B958. The step temperatures (degrees celsius) are indicated on the image

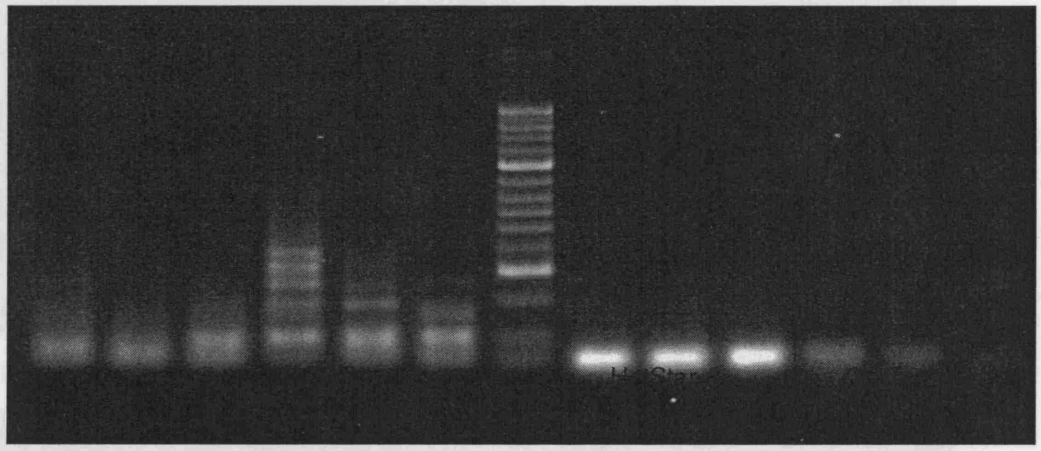


Fig 3.2 Examining the possible sources of error in PCR. (A) Neither the reagent kit used, whether HotStarTaq or Taq polymerase, nor the temperature at which the annealing step takes place appears to have any marked effect upon the β-globin PCR results for B958. There appears to be an EBNA1 product at 50C using the Taq kit, but this is very faint. **(B)** shows a similar result for both BZLF1 and LMP1. **(C) and (D)** Regardless of temperature, our BZLF1 and LMP1 primers still yielded no results. However, the alternative BZLF1 primer worked well, especially at the lower temperatures. This led to the stimulated B958 serial dilution of **(E)** where these primers are sensitive to a concentration of 100 cells.

BZLF1 (alternative)

50 52 55 61 65 68 HL2 50 52 55 61 65 68

Fig 3.3: Examining possible sources of error in PCR

Fig 3.3 C: Results for a temperature gradient PCR for B958 using an alternative primer for the same gene- BZLF1. Steps used (degrees celsius) are indicated on the image.

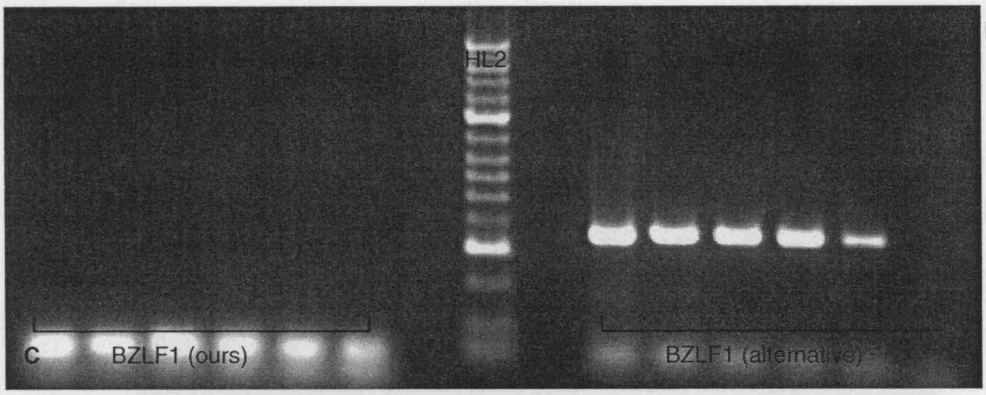


Fig 3.3 D: Results for a temperature gradient for B958 using the same LMP1 primer but different PCR reagent kits.

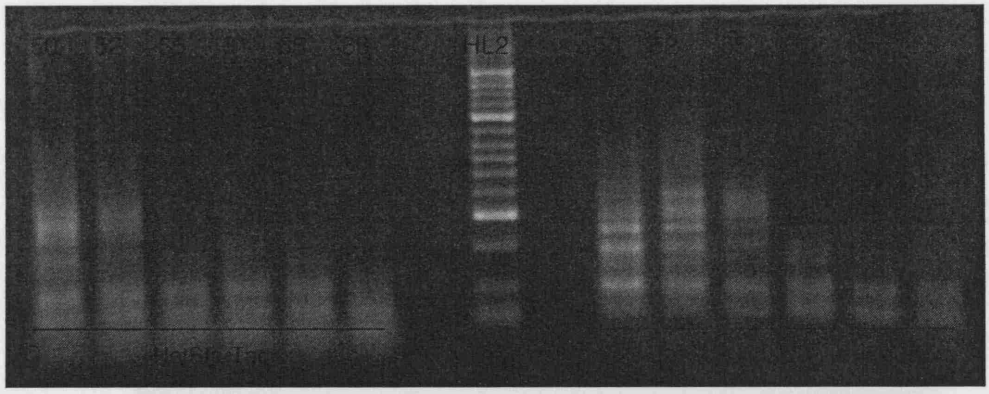


Fig 3.3 E: B958 Serial dilution PCR using alternative BZLF1 primer from fig 3.10C

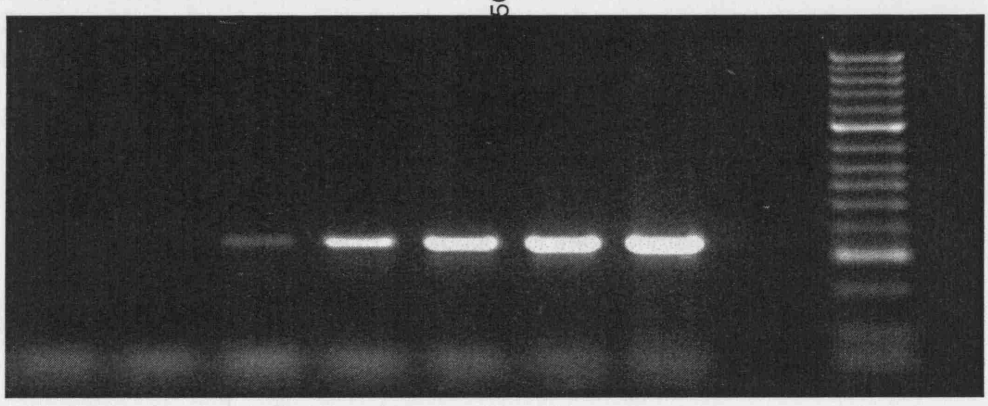


Fig 3.3 Examing the sources of error in PCR (C) and (D) Regardless of temperature, our BZLF1 and LMP1 primers still yielded no results. However, the alternative BZLF1 primer worked well, especially at the lower temperatures. This led to the stimulated B958 serial dilution of **(E)** where these primers are sensitive to a concentration of 100 cells.

Fig 3.3: Examining possible sources of error in PCR

Fig 3.3 F and G: PCR results from different EBV positive cell lines

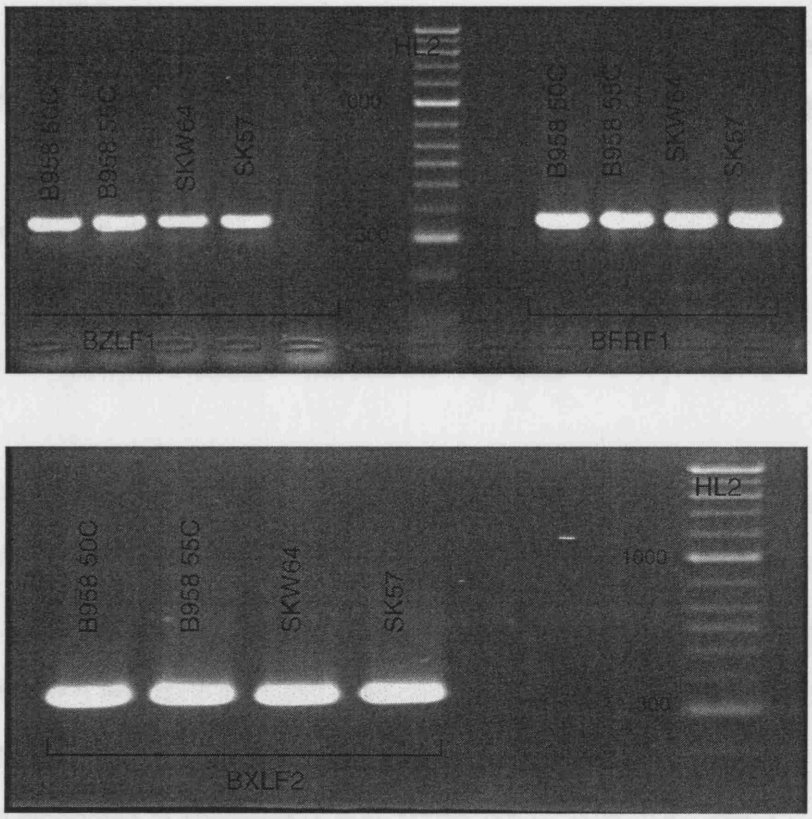


Fig 3.3: Examining the possible sources of error in PCR: Both figures (F) and (G) compare B958, our positive control for EBV, and two other lymphoblastoid cell lines (LCLs) known to contain EBV. These are SKW64 and SK57. The results show that all three cell lines are positive for the PCR products of these primers for BZLF1, BFRF1 and BXLF2. Two different temperatures were used for B958, simply as an alternative avenue of investigation, but the 5 degree difference appeared to have no effect on the strength of the resulting band.

effective at concentrations of 100 cells (fig 3.3 C, E and F) in different cell lines (fig 3.2 E-G). In order to test the quality of stimulated B958 DNA in these trials samples of EBV positive DNA from alternative cell lines were used for comparison. SKW64 and SK57 are both EBV transformed lymphoblastoid cell lines (Dr D Clark, UCL, personal communication). As well as the new BZLF1 primer that had proven effective, a further two entirely new primers were also used, BFRF1 and BXLF2 (both genes expressed early in lytic replication). All of these primers produced the same successful results regardless of the cell line responsible for the DNA, suggesting that this is not the source of the problem either (Fig 3.3 Fand G). These results do provide additional evidence for the fact that our positive control is an EBV containing cell line, as was stated previously. These results suggest that the primers used to test EBV infection in this cell line require further optimisation and were somehow degraded compared to initial standard curves within this cell line (Fig. 3.2).

Infection of epithelial cell lines with EBV. The immunostaining evidence suggests the successful EBV infection of the retinal pigment epithelium (RPE) and the bladder carcinoma (HTB-9) cells (Fig. 3.4 and 3.5). The pancytokeratin specifically stains the cytoskeleton (fig 3.4 A and B, and fig 3.5 A and B) in both cell lines confirmed their characteristic epithelial "cobblestone" morphologies. Both cell lines form single cell layers, though the RPE are more polarized and elongated in appearance than HTB9. When stained for EBNA1 the control cultures of both RPE and HTB9 do not show positivity (fig 3.4C, 3.5C). This agrees with PCR results which, whilst showing that there is definite presence of DNA as proven by the positive results for the β-globin primer in some time points, produce no result for the EBNA1 primers with the extracted control DNA from both epithelial cell lines (fig3.6A).

Figure 3.4: Infection of RPE Epithelial Cell Line with EBV

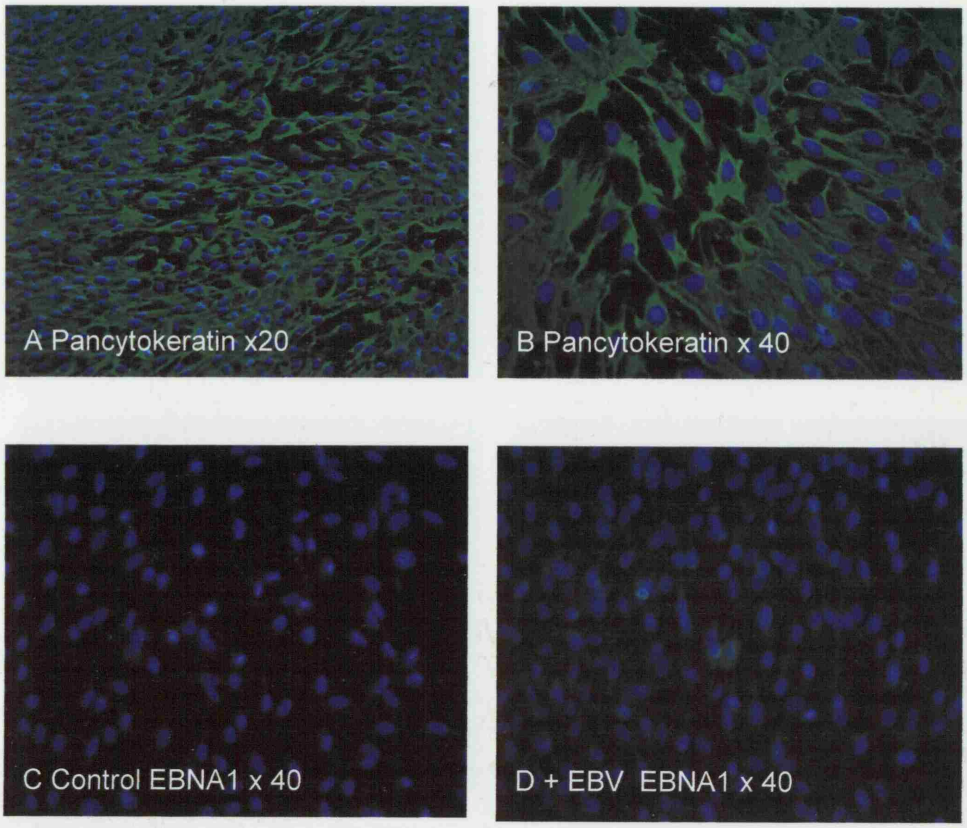


Fig 3.4 RPE infection with EBV (A) and **(B)** both show the characteristic 'carpet' of elongated and polarised RPE cells. **(C)** shows the lack of EBNA1 present in the cell line usually, whereas **(D)** shows EBNA1 positivity following infection with EBV. The negative .

Figure 3.5: Infection of HTB-9 Epithelial Cell line with EBV

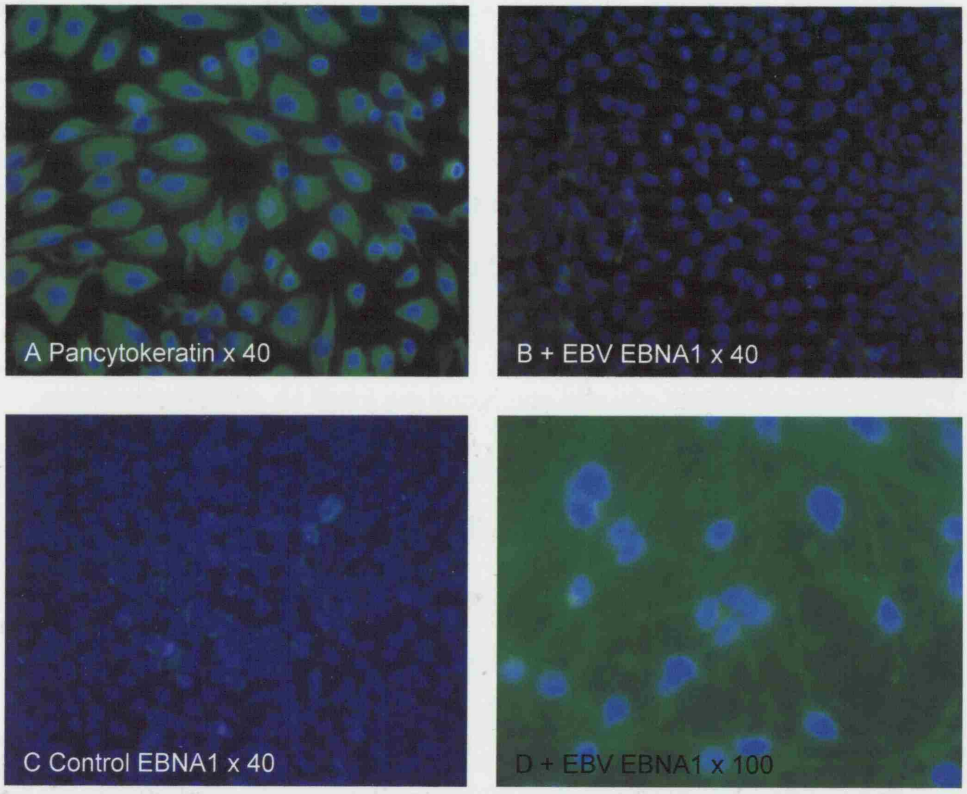


Fig 3.5 HTB-9 infection with EBV (A) Shows the epithelial nature of cell line expressing pan cytopkeratin. **(C)** shows a lack of EBNA1 in the control sample of the cell line, whereas after EBV infection it is visible **(B). (D)** shows this at a greater magnification, making the granular appearance of the stain apparent.

However, once infected with EBV there is clear indication of viral presence, as there is EBNA1 immunostaining in the EBV infected RPE and HTB-9 cells (fig 3.4D, 3.5B). This can be seen in even greater detail in the x100 magnification image of the HTB-9 infected cells, which reveals a granular appearance to the stain and its presence around the nucleus of the cells (fig 3.5D).

DNA extracted from the time series of the epithelial cell line EBV infection was tested using the same primers as these control samples. The HTB-9 samples did not give rise to β-globin PCR products although the control and EBV infected RPE did (Fig 3.6 A-B). The results for the EBNA1 PCR prove inconclusive, as there are some non-specific bands present in the RPE samples, but as there is no negative control reagent-primer mix in this PCR it is difficult to tell if a contamination could be responsible (fig 3.6C). However, the inconsistent signal fro B-globin within this series suggests that one of the confounding factors apart from PCR is that of sample degradation within this series of lysates.

EBV gene expression was also analysed by one step RT-PCR was used to test these cell lysates samples further, using the same primers to amplify specific transcripts from RNA extracted from the same time series. GAPDH was used instead of β-globin as the housekeeping gene (fig 3.7 A). GAPDH cDNA was present for all cell lines and infection points, including a positive control of stimulated B958 RNA. The blank negative control proves that there was no contamination in these samples due to the reaction mix.

However, when EBNA1 primers were used there was no result, even in the case of the positive control (fig 3.7B). The fact that GAPDH had shown conclusive results with the same samples suggests that it is not an RNA extraction issue, and the reagent mix was not contaminated. Even when repeated several times these results remained consistently negative (not shown).

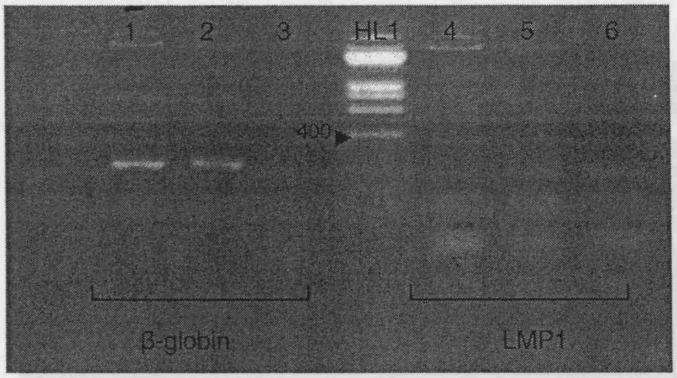


Fig. 3.6 A: β-globin and LMP1 PCR results for control epithelial cell lines

1. HTB-9 control
2. RPE control
3. Blank control of reagent-primer mix
4. HTB-9 control
5. RPE control
6. Blank control of reagent-primer mix

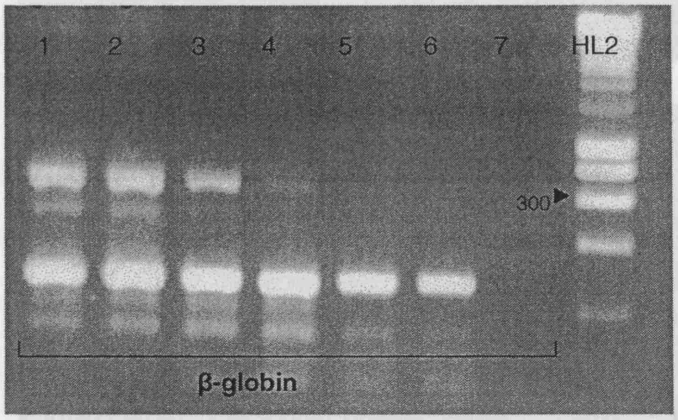
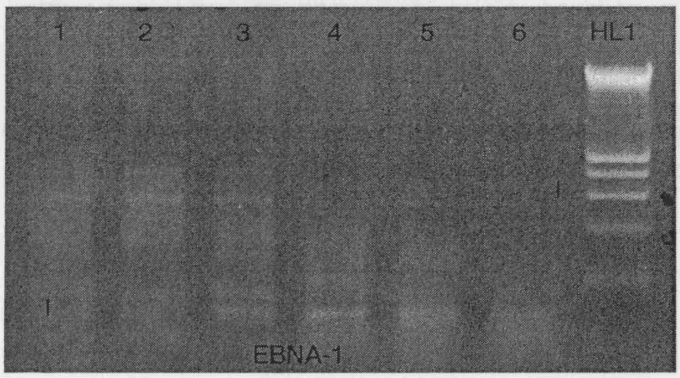


Fig. 3.6 B: β-globin PCR results for control and EBV infected epithelial cell lines at day 3 (d3) and day 6 (d6)

1. RPE control
2. RPE + EBV d3
3. RPE + EBV d6
4. HTB-9 control
5. HTB9 + EBV d3
6. HTB9 + EBV d6
7. Blank control of reagent-primer mix

Fig. 3.6 C: PCR results for control and EBV infected epithelial cell lines at d3 and d6.



1. RPE control
2. RPE + EBV d3
3. RPE + EBV d6
4. HTB-9 control
5. HTB-9 + EBV d3
6. HTB-9 + EBV d6

Fig 3.6: PCR results from epithelial cell lines RPE and HTB-9. (A) β-globin DNA was a PCR product from both RPE and HTB-9 control samples. Both cell lines proved negative for LMP1. **(B)** At d3 and d6 of infection β-globin DNA was detected in both the RPE and the HTB-9 cell lines. The blank control also shows no contamination. There are a large number of non-specific DNA fragments from the RPE PCR. **(C)** EBNA1 was not detected in either the control nor infected time points for both RPE and HTB-9.

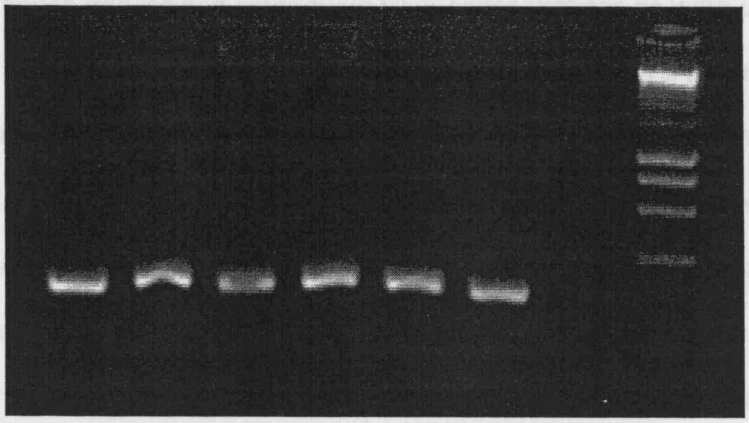


Fig. 3.7 A: RT-PCR results for epithelial cell lines control and EBV infected d3 and d6

1. HTB9 control
2. HTB9 + EBV d3
3. HTB9 + EBV d6
4. RPE + EBV d3
5. RPE + EBV d6
6. Stimulated B958
7. Blank control reagent mix

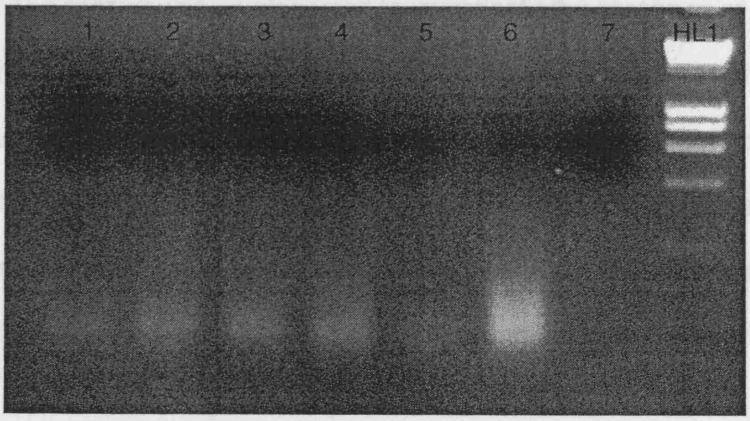


Fig 3.7 B: RT-PCR results for epithelial cell lines control and EBV infected d3 and d6

1. HTB9 control
2. HTB9 + EBV d3
3. HTB9 + EBV d6
4. RPE + EBV d3
5. RPE + EBV d6
6. Stimulated B958
7. Blank control reagent mix

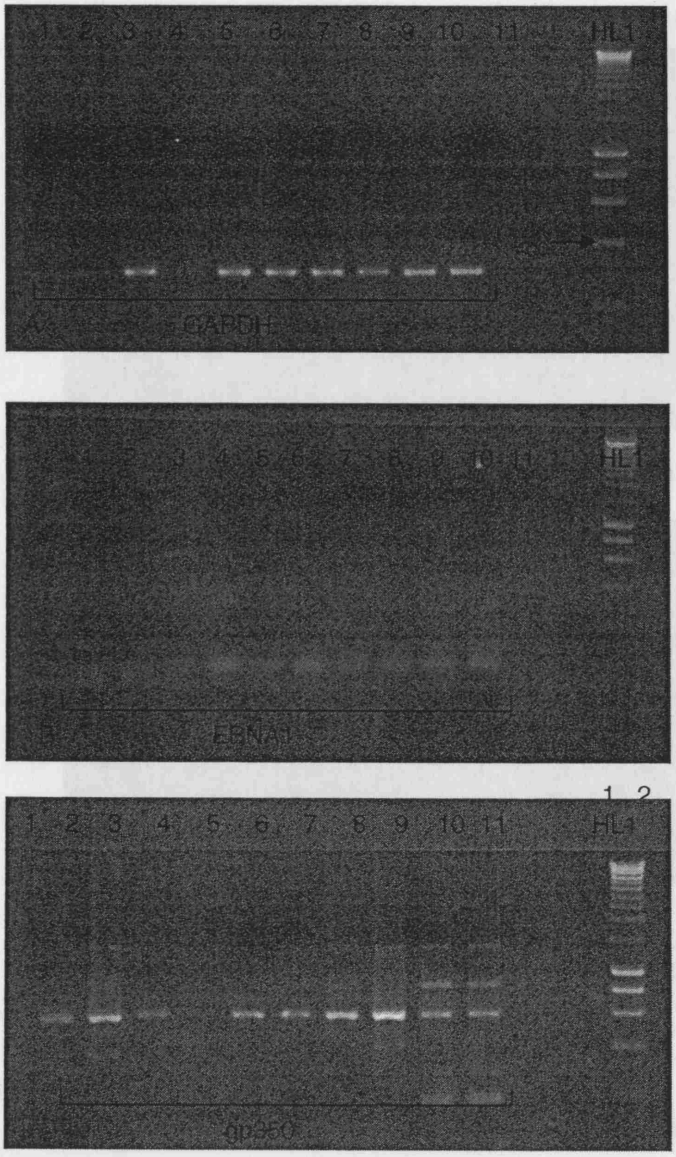
Fig 3.7: One Step RT-PCR results from epithelial cell lines RPE and HTB-9. (A) Although the molecular marker (HyperLadder I) appears faint on this image, it is still possible to see positive results for all cell sample extracted RNA using the GAPDH primer. This produces an amplicon of 268bp. The negative control of the reagent-primer mix is also clear, indicating that there is no contamination. However, **(B)** shows no EBNA1 primed PCR products from the extracted RNA, even in the positive control stimulated B958 cell line.

Two-step RT-PCR was then introduced to test these infected epithelial cell lines. Cultures from days 12 and 26 from another repetition of the same time series were investigated. The RNA extraction from the earlier time points of d3, d6 etc, in this repeated infection series had yielded inadequate RNA concentrations so were unavailable. As was the case for the one step PCR of the earlier time points (fig 3.7), the use of the GAPDH primers results in the presence of amplified GAPDH cDNA in both control and infected epithelial cell lines at days 12 and 26, as well as the positive controls of stimulated and unstimulated B958 (fig 3.8A) but in a more inconsistent manner compared to previous. These variable results are suggestive of RNA degradation in this time series.

The use of the primers for EBNA1 (fig 3.8 B) once again proved unsuccessful for these RNA samples. The technique was also used to investigate the presence of a lytic cycle specific transcript using the gp350 primers. The results (fig 3.8 C) were negative for all the epithelial cell samples, although there is a faint band in the B958 positive control as would be expected. Many non-specific bands, one especially prominent in all samples but day 26 of the epithelial infection, were also present. However, in this reaction the negative master mix control also showed these non-specific bands, suggesting contamination of the amplification process.

Due to a greater result for HTB-9 in these two-step PCRs, it was tested again in isolation with GAPDH and LMP1 primers (data not shown). Again there is evidence of amplified GAPDH cDNA (Fig 3.8 D). These results suggest that EBV was present at a low level in these cell lines, but further work is necessary in order to detect RNA transcripts consistently across the time series.

EBV infection of human mixed brain cultures. The PCR results from human mixed brain cultures show samples infected with EBV harvested from B958 and HTB9 at day 7 post infection (fig 3.9). Using one step RT-PCR for EBNA1 proved to be a success by using freshly manufactured primers (fig3.9). In these



C

Fig. 3.8 A, B and C: two step RT-PCR results from control and EBV infected epithelial cell lines at d12 and d26

1. RPE control d12

2. RPE + EBV d12

3. RPE control d26

4. RPE + EBV d26

5. HTB9 control d12

6. HTB9 + EBV d12

7. HTB9 control d26

8. HTB9 + EBV d26

9. Unstimulated B958

10. Stimulated B958

11. Blank reagent primer mix

1 2 3 4 5 6 7 8 9 10 11

Fig 3.8: Two step RT-PCR results from control epithelial cell lines, and at d12 and d26 of EBV infection: (A) shows evidence of GAPDH transcripts in all cell lines, though the results are very faint for RPE. There is also no reagent mixture contamination. **(B)** The EBNA1 primers produced no valid results, even in the EBV positive control cell lines of stimulated and unstimulated B958. **(C)** There no visible band to correlate with the correct amplicon size of 2582bp in the gp350 PCR, but there are fragments visible in the positive controls that are also seen in the epithelial cells, both EBV infected and control samples.

Fig 3.9 A OneStep RT-PCR from mixed brain cultures d7 EBV infection- EBNA1.

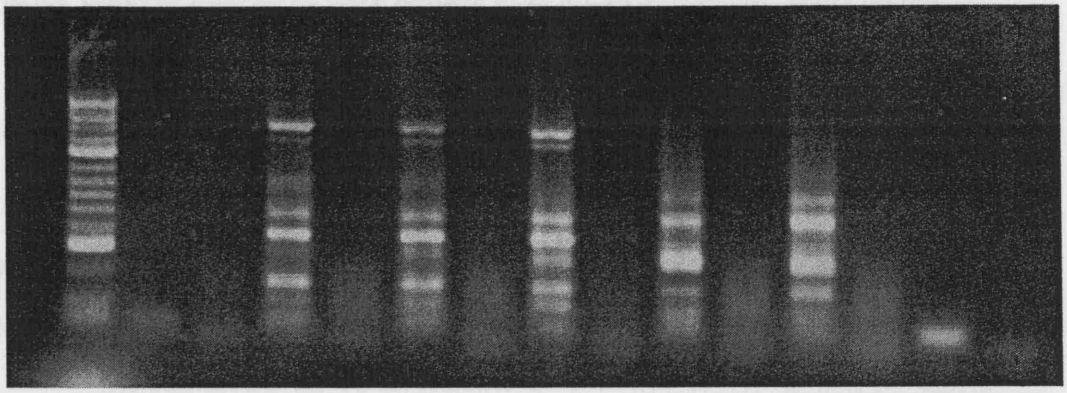
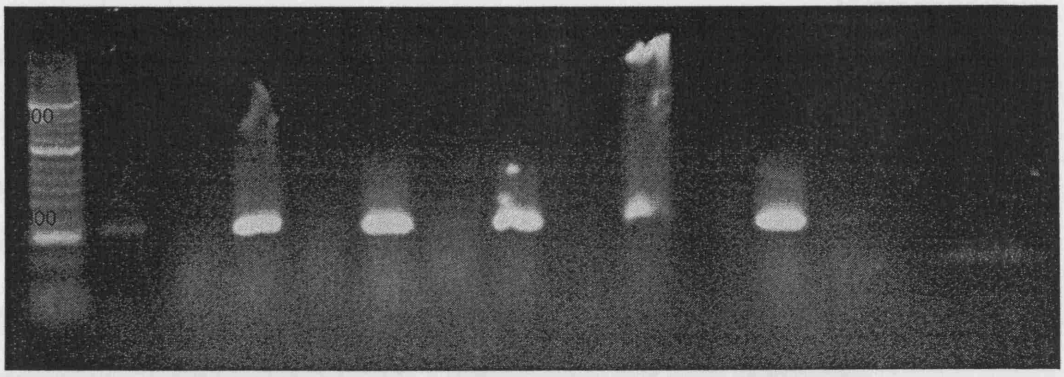


Fig 3.9 B OneStep RT-PCR from mixed brain cultures d7 EBV infection- GAPDH



1. Control MC; 2. Control MC RT –ve; 3. RPE EBV MC; 4. RPE EBV MC RT –ve; 5. HTB-9 EBV MC; 6. HTB-9 EBV MC RT –ve; 7. B95-8 EBV MC; 8. B95-8 EBV MC –ve; 9. Stimulated B958; 10 Stimulated B958 RT –ve; 11. Unstimulated B958; 12. Unstimulated B958 RT –ve; 13. H_2O; 14. H_20 RT -ve

Fig 3.9 RT-PCR results from Mixed Brain Cultures (MC)

(A) These results indicate that the B958, RPE and HTB9 EBV infected MC do express EBNA1, present as a band at 1795bp in all three MC samples, and absent in the MC control sample RT-PCR (lane 1). This band is absent from the positive control samples of B958, both stimulated and unstimulated. This is likely due to a difference in the nature of the majority splice product specifically found in these cells. **(B)** shows the GAPDH RT-PCR results from d7 of a 21 day infection series. There is a result for each culture type, and the RT lanes are clear with the exception of those for HTB-9 and RPE EBV MC (4 and 6).

reactions RT step negative samples were prepared alongside to investigate any DNA contamination in the samples that had not been removed during the DNase step in the RNA extraction protocol. GAPDH transcripts were present in all RT-PCR cultures, although the result for the mixed culture control is weak in comparison (fig 3.9A). The most promising result is the presence of EBNA1 transcripts in the mixed brain cultures infected by EBV derived from B958, HTB-9 or RPE (fig 3.9A). It is likely that the absence of this 1,795bp fragment in both the stimulated and unstimulated B958 positive controls is due to the presence of multiple splice variants of EBNA1 in this cell line, although this requires further investigation.

The results of the Mixed Culture immunocytochemistry support the presence of EBNA1 at the protein level in this culture system as well as at the RNA from the RT-PCR (fig 3.10). There is evidence of EBV infection when the cultures are exposed to EBV from all three sources (i.e RPE, HTB-9 and B95-8). EBNA1 positive cells appear at d3 post-infection, a difference emphasized by the complete absence of EBNA1 signal in the control mixed cultures. In comparison to the control (fig 3.11) it is possible to see EBV distribution throughout cells in the culture (fig 3.11A-B). There seems to be no quantitative or qualitative difference in the images of infection arising from EBV from differing cell lineages whether B cell or epithethelial.

The specific immunostaining of the astrocytes through GFAP (fig 3.11A-C), and the neurons by β-tubulin III (fig 3.11D- F) show the dense network of the cells in this primary system. The impact of EBV in other cell populations, such as oligodendrocytes and microglia requires further study.

EBV infection of human neuronal cultures. Following the successful infection of the Primary Human Mixed Brain Cultures with EBV as described above. EBV infection with virus produced in B958, RPE or HTB9 cells was also found to culminate in EBNA1 punctate expression in the Primary Neuronal Cultures through

Figure 3.10 A: Mixed Culture Infected with EBV from different cell types

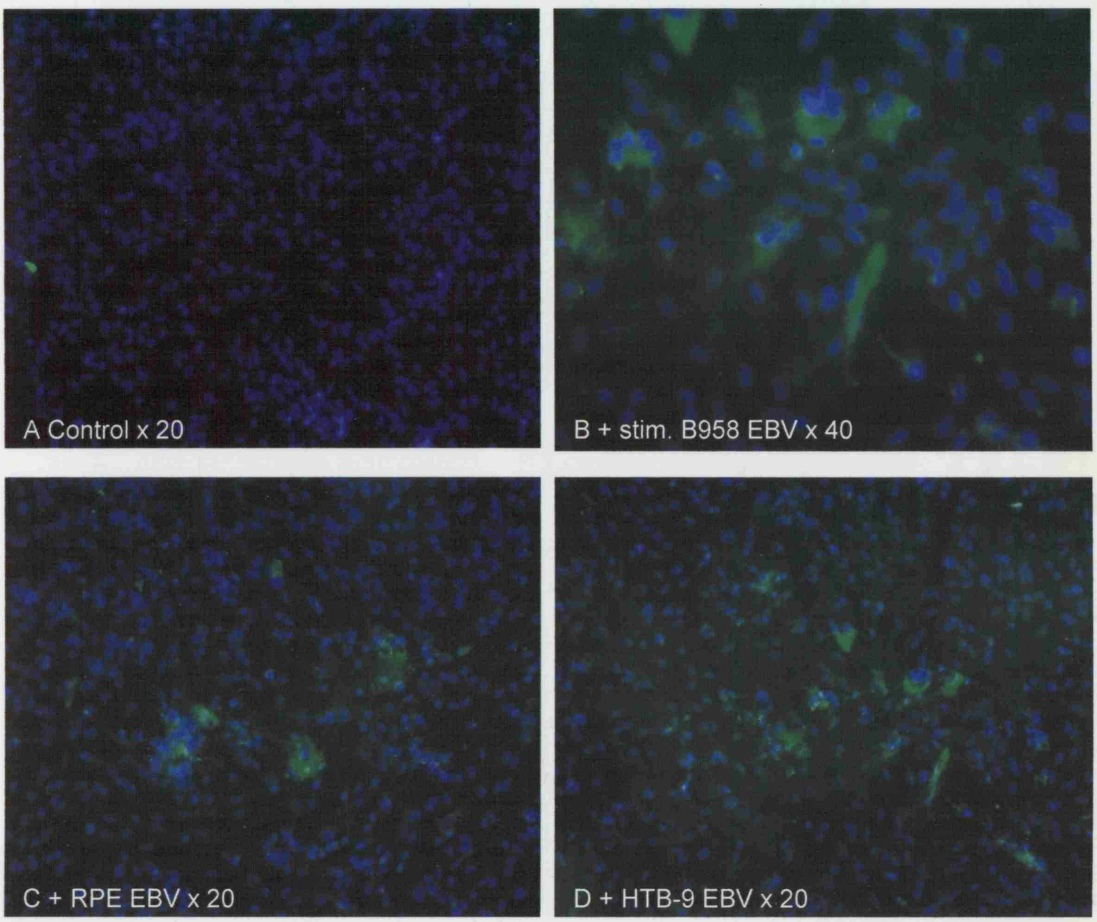


Fig 3.10 A: In comparison to the level of EBNA1 positive staining seen in the control sample, it would appear that there is considerable difference in the results for the Mixed Culture samples infected with EBV. This remains true regardless of the source of the virus- whether harvested from B958 **(B),** RPE **(C)** or HTB9 **(D).**

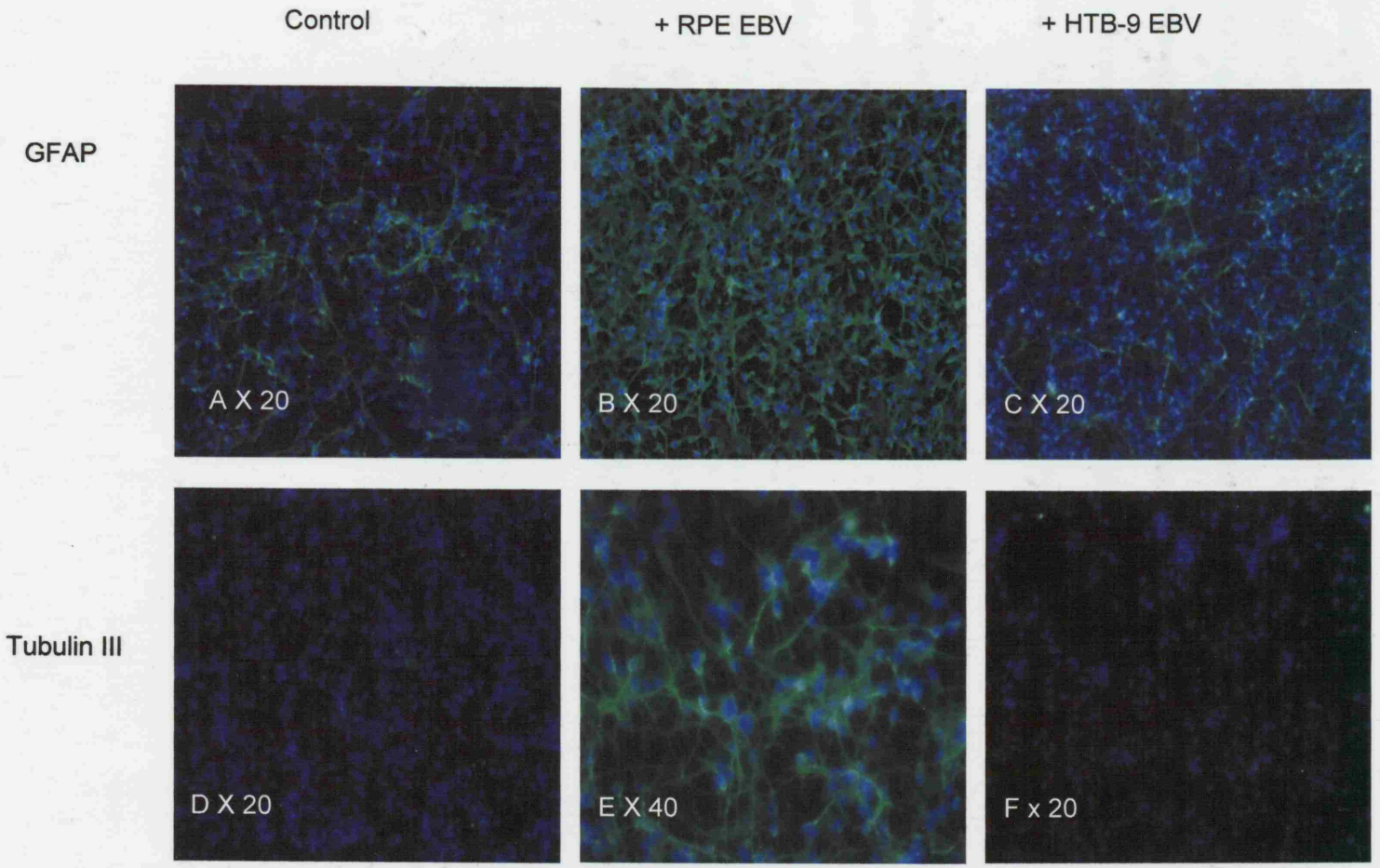


Figure 3.11 Human Mixed Brain Culture Infection Control

Fig 3.11 B: Mixed Cultures: these results show the compositional elements of the mixed cultures, both in a control state and after EBV infection from RPE and HTB9. The Tubulin III stain has not been so effective in the control (D) and HTB9 EBV infected (F), making it difficult to judge if there has been a change in cellular population following infection, or even a change in cell morphology. The same is true of the glial fibrillary acidic protein (GFAP) results.

immunostaining. These results demonstrate that EBV infection culminates in viral protein production within this system 3 days after infection. These results require further validation at the RNA level for viral transcripts. In these cultures, Myelin associated glycoprotein (MAG) was used as a marker for oligodendrocytes (fig 3.12A,B), GFAP for the astrocytes (Fig3.12C, D) and Microtubule Associated Protein 2 (MAP2)(fig3.12CE, F) was used to identify the neurons. In comparison to the control results for the mixed brain cultures it is possible to see how the oligodendroctye cytoskeletal density is reduced in these neuronal cultures when infected with EBV from HTB-9 at day 7 post infection (fig 3.12 A,B).

In comparison with the control staining for EBNA1 all infected samples tested showed EBNA1 positivity, regardless of the source of the virus. At the highest magnification of x100 it is possible to see punctuate staining present along the cell processes (fig3.12B,D). These results are preliminary but suggest that it is possible for EBV to infect human brain cell cultures culminating in viral protein expression and viral gene expression within these primary systems.

Fig 3.12 Neuronal Culture infected with EBV

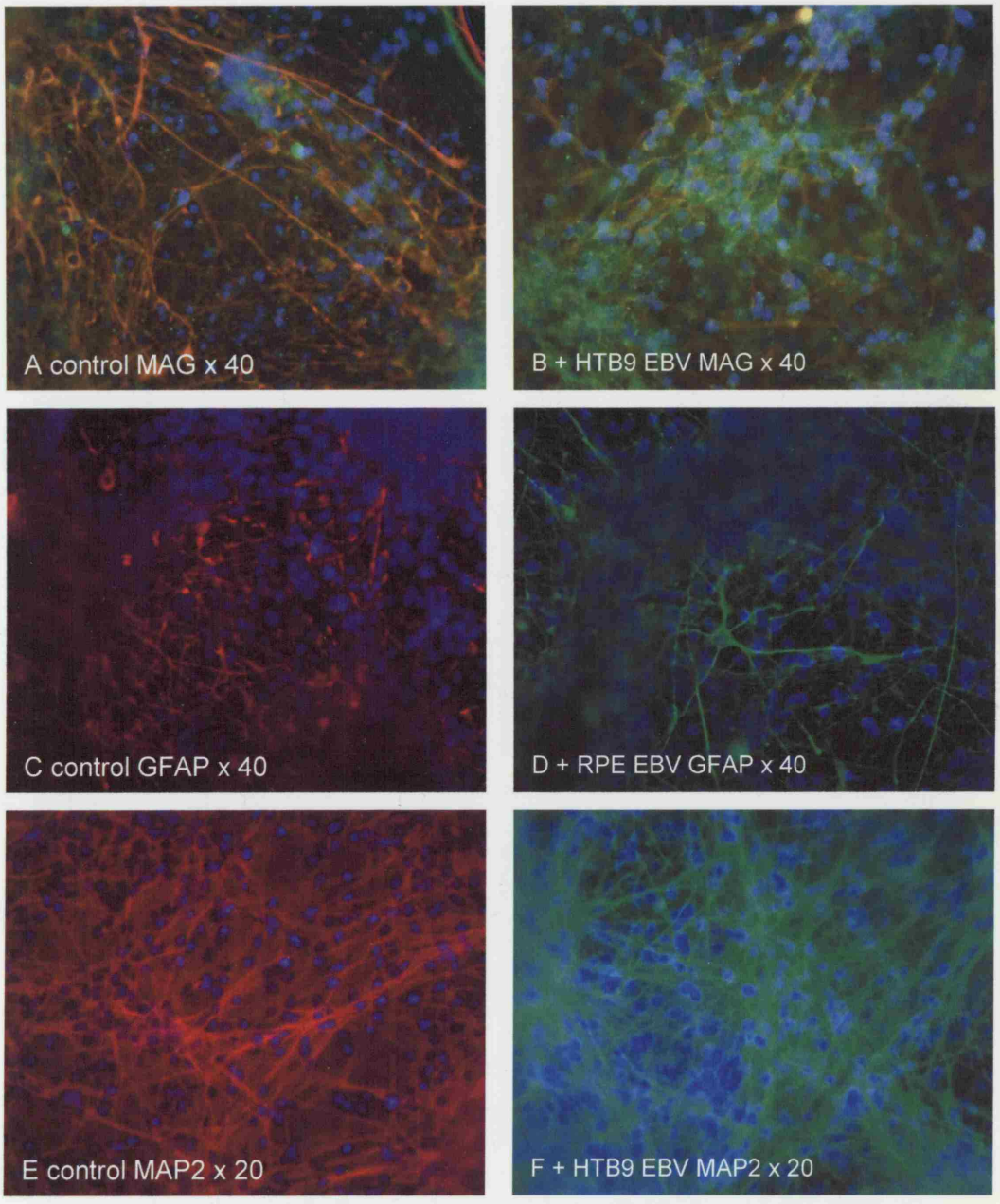


Fig 3.12 A: Neuronal Cultures. (A) and (B) show control and EBV infected neuronal cultures stained for MAG, which will indicate oligodendrocytes. **(C) and (D)** show the results of GFAP staining for the control and EBV infected cultures, and **(E) and (F)** are from Microtubule Associated Protein 2 (MAP2) staining which indicates neuronal dendrites. Using these staining techniques there appears to be no obvious difference in the cellular composition of the neuronal cultures, or the morphology of the cells themselves.

Fig 3.12 B Neuronal Culture infected with EBV

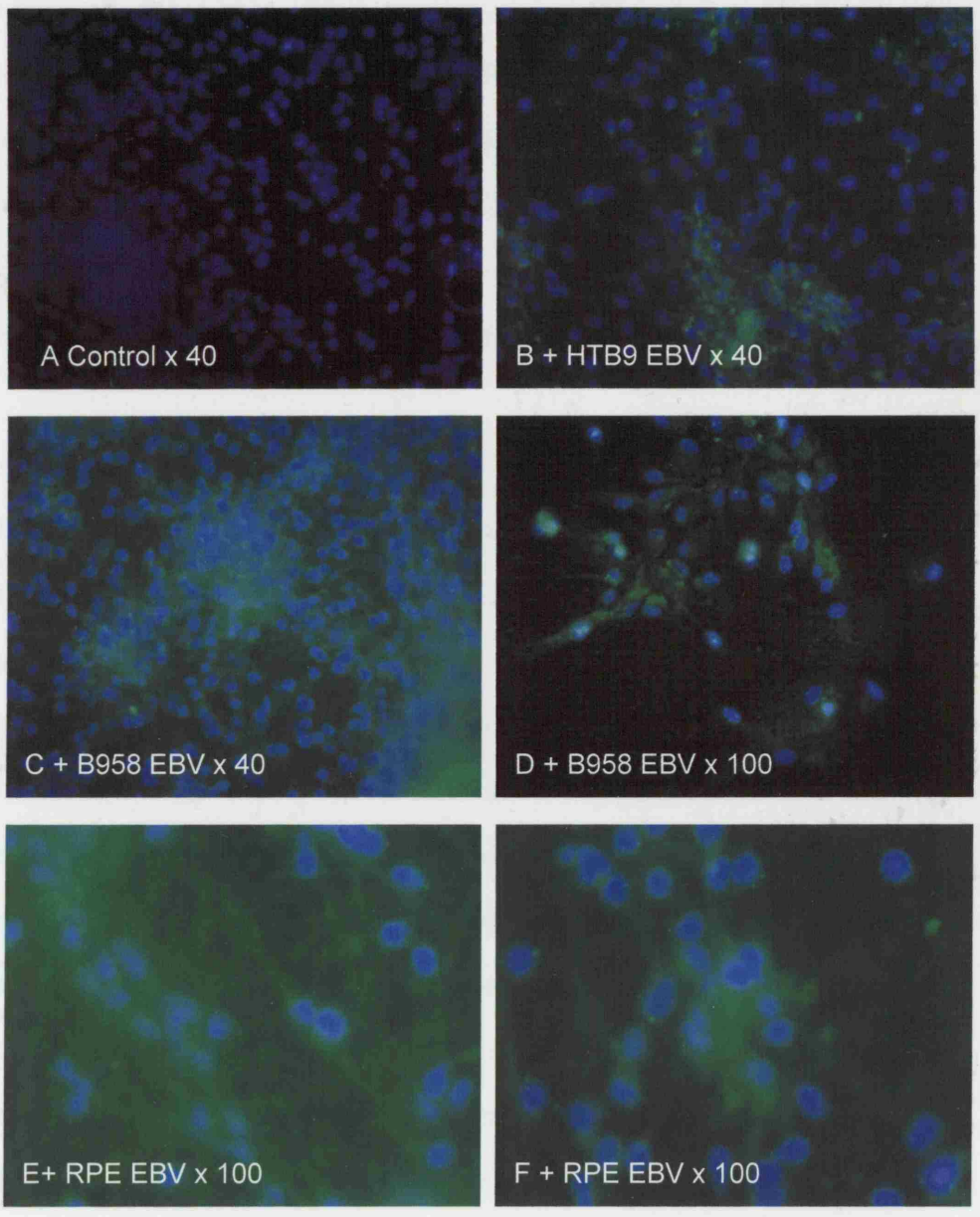


Fig 3.12 B: Neuronal Cultures plus EBV from B958 (C) and (D), RPE (E) and (F) and HTB-9 (B) sources: Despite the differing sources of EBV, these images show EBNA1 positivity with the same granular presentation, suggesting the presence of viral particles. This is especially evident in **(D)**, and in the other images at the higher magnification.

4. Discussion

This study was designed to test if Human brain cells can be infected with EBV as a neurovirological model of MS. The virus was grown and harvested from a synthetically engineered B cell line, B95-8, and the cell free virus produced by these cells was used to infect retinal pigment epithelial (RPE) and Human Bladder Carcinoma (HTB-9) cells. The productive infection of the epithelial cultures resulted in an alternative source for EBV. Virus derived from these epithelial cells and directly from the B cells was then used to infect mixed brain cultures as a possible model of white matter EBV infection, and neuronal cultures as a model of grey matter EBV infection.

The results from both primary culture systems suggest that EBV infection of these CNS tissue models is indeed possible, and that it can be achieved when the virus has been harvested from RPE or HTB-9 epithelial cell lines, or from B95-8 lymphocytes. This also supports the successful infection of these epithelial cell lines. To the best of our knowledge no previous research exists that has shown EBV infection in primary brain cells in culture, and our observations are in direct opposition to previous work with RPE which did not observe any productive EBV infection (Huemer et al. 1996). However, timing of infection and EBV dose used for the infection varies widely from study to study and this could offer an explanation for our observed low level of infection in this cell line. Another bladder carcinoma cell line, T24, has been shown to be susceptible to EBV infection in vitro, though not through exposure to cell-free virus (Imai, Nishikawa, & Takada 1998).

The intention was to follow the infection over 2 or 3 weeks as it is known that the program of EBV gene expression that dictates the level and nature of infection varies due to time after infection as well as the lineage of host cells. From studying specific time points during this period (3-26 days post-infection) attempts were made to not

only prove that the virus could infect the cultures, but also to characterize the expression profile of EBV in each culture type at each time point with the expectation that this would differ. Specifically this involved determining if EBV DNA is present in infected cells when compared to controls. We found evidence for a low level expression of EBNA1 in infected cultures (fig 3.4, 3.5). However, due to various technical difficulties involving EBV gene splicing mechanisms, as well as primer and sample stability, we were not able to obtain a consistent signal as proof of EBV infection at the DNA or RNA level within this epithelial system. Nevertheless, this warrants further investigation.

The failure of the majority of the PCRs and RT-PCRs did have a large impact on the intended design. For example, originally it was the epithelial line shown to be most virally productive that was to be used to infect the primary cultures. Since we lacked this information both were used. A large amount of time was spent on repetition of experiments, and attempts to identify the source of problems that encompassed most aspects of the experimental process- from the maintenance of the cultures themselves through to the PCR.

The PCR result of most value is the proof that EBV DNA is not present in the control samples of the epithelial cell lines, a claim that has additional support through the lack of EBNA1 specific staining during immunocytochemistry with these cultures. PCR also proved that our positive control of stimulated B958 cells *were* EBV positive, and genes involved in both latent and lytic programmes could be detected. Similar successful results for the unstimulated cell line would be useful, but all attempts at this failed. These would give a point of comparison with the activated and inactivated cells, rather than with just the staining in isolation.

This approach, within the context of EBV, is exemplified in the Menet et al paper (1999), where viral infection of transfected astrocytes was followed over a period of up to 62 days. Their mRNA analysis revealed the change in gene expression over this time in astrocytes, from an initial lytic period to a unique latency within the first 13 days of this period. This restricted type of infection illustrates the difficulty in detecting EBV RNA transcripts in our primary brain cell culture systems.

The fact that there is also a difference according to the cell type in which the virus is resident is supported by RT-PCR results for the mixed brain cultures (fig 3.10). These are limited currently to a single time point of day 7 post infection, but show an amplified product for EBNA1 indicating that there is successful EBV infection established in these cultures. This is true regardless of whether the virus used to infect these cultures is sourced from the EBV containing B958, RPE or HTB9. The positive immunostaining results for EBNA1 in these cultures provide support for these findings at the protein level (fig 3.10).

These RT-PCR results for the mixed brain cultures also provide evidence for differences in the pattern of viral transcription between the B958 cells and the mixed brain cultures (fig 3.9). There are several bands that appear for both the stimulated and unstimulated B958 cells that are not seen in the mixed cultures. It is likely that these correlate to a greater presence of a smaller EBNA1 splice variant in these highly virally productive cells. This would also offer explanation for the lack of the expected 1,795bp fragment in these control samples. In order to test this hypothesis the fragment would need to be excised from the gel and sequenced. It remains to be seen if these results can be duplicated in the neuronal cultures, but these results suggest that we would expect to see a similar difference between the viral activity in this primary system and the B958 cells.

The observation of EBNA1 Ab positivity in epithelial and primary culture immunocytochemistry and the RT-PCR results that show its expression in the mixed brain cultures are sufficient to indicate successful viral presence in these cultures, but still reveal nothing of the nature of the EBV infection. In order to make this a comprehensive study that would truly prove the dynamics of EBV infection in these epithelial cell lines and CNS tissue there needs to be successful examination of every time point in the series for every culture. This would require RT-PCR for the latent and lytic EBV genes and accompanying immunocytochemistry, and comparison of these results with our positive control of B958. From this point onwards there are several additional experimental procedures that would support the argument for EBV as a neurotropic virus.

A further technique that was discussed was the use of cell culture insert plates to promote viral infection through cell-to-cell contact between the B cells, epithelial cells and the primary cultures. Imai et al (1997) found this to be the most efficient means of EBV infection in several epithelial cell lines, exhibiting an 800% increase. This would certainly be worthy of investigation in the mixed brain and neuronal cultures rather than just exposure to the cell-free virus. It would allow for the promotion of more 'normal' behaviour; both the apical and basolateral sides of the cells will have access to the medium, and *in vivo* it is likely that the brain tissue is not the primary site of infection and thus would not be in contact with cell-free virus.

Western blot analysis would also prove useful in combination with the proof at the RNA level. This is a method by which proteins present in the culture samples can be denatured and then separated by mass. These separated proteins are consequently transferred out of the gel onto a nitrocellulose membrane, where they can be probed and identified by Abs specific to the protein under investigation. In this case the presence of EBV proteins would be the desirable result.

An additional area of immediate interest is the virus receptor, complement receptor 2 (CR2) or CD21. Although in the context of EBV infection in patients it has been excluded as a reason for long-term seronegativity in individuals (Jabs et al. 1999), and certain epithelial cells can be infected in the absence of CD21, it is still the main receptor associated with EBV access to the cell (Turk et al. 2006). Astrocytes have been shown to be CR2 positive, and it is claimed that these are the only brain cells that express them (Gasque et al. 1996). Whether or not this is true of other cellular components of the primary cultures would be another avenue of investigation, and if CR2 is indeed absent, what is the mechanism of their successful infection?

Within a culture system some cell types are often preferentially infected. The presence of several specific cell types, with characteristic anatomical and physiological features implies that this is true of these mixed and neuronal cultures. In support of this aspect of the infection the immunocytochemistry could have been improved through the extended use of triple immunolabelling in all cultures. This would allow for the identification of co-localized Abs, for example EBNA-1 with CD21. This would allow for visualization of relationships between the virus and specific cell types, locations or receptors.

Which cell types are the most susceptible to infection, and how the viral expression profile differs between and within the cells may be of particular interest in the case of linking EBV to MS, where the pathology is primarily associated with oligodendrocytes. In our system, paucity of oligodendrocyte processes upon EBV infection in the primary Human neuronal system (fig 3.12) are indicative of specific oligodendrocytic damage and warrant further investigation.

The difference in viral expression observed by RT-PCR between the mixed cultures and B95-8 support the fact that there are variations dictated by the cell type, as does

the unique profile that Menet et al (1999) described in the EBV infected astrocyte cultures. This involved a latent period where Rta, the product of BRLF1, functioned as the transactivator rather than ZEBRA, the product of early lytic gene BZLF1 that is usually responsible for stimulating the expression of lytic genes. This has also been seen in epithelial cells (Ragoczy, Heston, & Miller 1998).

Since EBV DNA has been found in the CSF of MS patients, as well as in biopsy samples of brain tissue, it is possible that in these cases EBV has made contact with CNS cells. Our results for both RT-PCR and immunocytochemistry support the idea that it is possible for EBV can infect these cells. However, this still does not address the issue- EBV may well be a neurotropic virus, but how much evidence does this provide for the role of EBV in MS?

There are still many questions that remain unanswered about EBV and MS, despite the length of time that the two have been linked. The observed increased Ab titers may be due to the fact that MS patients are immunocompromised as the high seropositivity is not a detail exclusive to MS- over 99% of systemic lupus patients are EBV positive (Christensen 2006). A recent hypothesis suggests that EBV infection is instrumental in the development of MS in a synergistic manner, and one of the most important factors in controlling the immune system response to EBV infection is the vitamin D status of the individual (Holmoy 2007).

MS is a very heterogeneous condition and there are many theories of the mechanisms behind its pathogenesis and progression. As with any disease, it is the understanding that will undoubtedly be instrumental in triumphant clinical treatment. Although the successful infection of these primary cultures with EBV does not prove without doubt that it is involved in the pathogenesis of MS, it will provide a valuable

model of the pathology of CNS infection that may contribute to the understanding of just how instrumental EBV is in the cellular pathology of MS.

Conclusion

In conclusion, these results suggest that EBV may be able to infect primary human brain cells in culture. We have shown that EBNA1 expression is present in B cells (fig3.1) as well as in epithelial cell lines RPE and HTB-9 (fig 3.4, 3.5) and primary human mixed brain (fig3.10) and neuronal cultures (fig 3.12) when they are exposed to cell-free EBV. There is also evidence that supports the production of different EBNA1 splice variants in the EBV infected human mixed brain cultures as compared to the B cell B95-8 EBV system (fig 3.9). However, these are preliminary results that require further investigation.

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